Site specific cross-linking of thio-nucleosides

Thesis

SITE SPECIFIC CROSS-LINKING OF THIO-NUCLEOSIDES

By

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ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cdks	Cyclin-dependent kinases
Rb	Retinoblastoma
UV	Ultraviolet
IR	Infrared
ROS	Reactive oxygen species
BCC	Basal cell carcinoma
SCC	Squamous cell carcinoma
CMM	Cutaneous malignant melanoma
DPCs	DNA protein cross-links
THF	Tetrahydrofuran
Et_3N	Triethylamine
T^{SDNP}	S^4-2,4 Dinitrophenylthiothymidine
T^S	4-Thiothymidine
O.N	Overnight
FDNB	1-Floro-2,4-dinitrobenzene
ClDNB	1-Chloro 2,4-dinitrobenzene
1F2NB	1-Fluoro-2-nitrobenzene
1F4NB	1-Fluoro-4-nitrobenzene
T	Thymidine
nm	Nanometers
dR	2´-Deoxy ribose sugar group
NTs	Nucleoside Transporters

DTT	Dithiothreitol
S^4BrdU	4-Thio-5-bromo-deoxyuridine
S^4BrdU^{DNP}	S^4-2,4-dinitrophenyl-5-bromodeoxyuridine
CPG	controlled porous glass
min	minutes
s	seconds
T^{TRI}	4-Triazolothymidine
T^{CYS}	4-Cysteinylthymidine
$T^{CYS\text{-}CYS}T$	Dimer of 4-cysteinylthymidine
T^{GLU}	4-(S-Glutathionyl)-thymidine
S^4-2,4dnpBrdU	S^4-2,4-dinitrophenyl-5-bromodeoxyuridine
DNP	Dinitrophenyl

ABSTRACT

Cancer is a devastating disease. Numerous drugs have been synthesised over the years aiming to cure cancer. Significant new advancements in anti-cancer therapy have been achieved over the past few years. This was made possible by the molecular level understanding of the cancer disease, which has led to the synthesis of various targeted drugs.

The targets within the cancer cells are very limited for anti-cancer drug design because cancerous cells resemble normal cells in most of their molecular aspects. This makes it extremely difficult for cell specific incorporation of drugs or cell killing. So, alternative approaches have been followed for specific cell killing. However, such approaches are still at an early stage and a great deal of work needs to be done. Various DNA-targeted approaches, such as photo cross-linking and chemical cross-linking; have been developed for killing cancerous cells. These approaches have not completely solved the problem of specificity. Like traditional anti cancer drugs, these agents also indiscriminately cross-link DNA in both normal cells, cancerous cells leading to cell death.

In this thesis, two modified nucleosides were chosen which can be incorporated into the DNA of the cells easily. These thio-modified nucleosides were proven to have more affinity towards the DNA of the cancerous cells. Such modified nucleosides have previously been exploited for anti-cancer activity, especially UVA cell killing. In this thesis the thio-modified nucleosides have been explored for their site specific cross-linking activity.

The first modified nucleoside 4-thiothymidine was synthesised and reported in 1959. 4-Thiothymidine resembles thymidine, except for a modification at position 4 with sulphur instead of oxygen. Previous studies have shown that 4-thiothymidine behaves

like thymidine when incorporated into the cells. Like thymidine, this is base-paired with adenine in DNA. In this thesis, at first the thiol group of 4-thiothymidine has been specifically activated by a stable and readily replaceable group. This group was then replaced by various thio-nucleophiles which lead to the cross-linking of the nucleoside. The standards of these cross-linked nucleosides were successfully synthesised and characterised.

In the quest to improve specificity of incorporation into cancer cells a novel pro-drug, 4-thio-5-bromodeoxyuridine (S^4-BrdU) was synthesised by members of our group in 2003. This nucleoside is another thymidine analogue which is modified by replacing oxygen at position 4 with sulphur and methyl at position 5 with bromine. The thiol group of S^4-BrdU nucleoside was chosen for the purpose of cross-linking.

Successful cross-linking of the nucleoside with various thiol nucleophiles has been explored. Firstly S^4-BrdU was converted into a DNP derivative. The DNP was then replaced with mercaptoethanol, thioethanethiol and natural thiols such as cysteine (the only amino acid containing a thiol group in the molecule) and glutathione (a natural peptide which helps in detoxification pathways in the body). These cross-linked products have been characterised by various methods.

After successfully cross-linking the nucleosides, oligomers containing 4-thiothymidine were synthesised. The oligomers were characterised and 4-thiothymidine was successfully incorporated into the oligomers.

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CHAPTER-*1*

INTRODUCTION

1. Introduction

1.1 The Cell

The basic units of all living organisms are called cells and all cells undergo the same basic biological processes. Cells have the following common main features:

- A plasma membrane which encloses and defines the cell and
- DNA which contains genetic information for biological processes
- Transcribing the information from DNA into RNA
- Translation of RNA to proteins
- Synthesising of proteins using the same set of 20 amino acids [1].

1.2 Cell Structure

A cell contains various organelles inside the plasma membrane such as the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, peroxisomes, vacuoles etc. The nucleus is surrounded by a double membrane called the nuclear envelope, inside of which is DNA, the genetic material. The nuclear envelope differentiates the processes that occur inside the nucleus, DNA replication and synthesis of RNA from the processes that occur in the cytoplasm, translation of RNA to protein.

1.3 Organisation of DNA into Chromosomes

DNA is a long, un-branched, linear polymer that contains millions of nucleotides in non-random fashion; the genetic information is stored in the exact order of the nucleotides. The genetic code is written in words of three nucleotides. Only a small portion of the DNA codes for proteins and is called as coding DNA. Functions of non-coding DNA are not fully understood but they contain sequences for other biological

functions, such as patterns of DNA folding in each chromosome. DNA folding is not only important in packaging of the long DNA molecules in an orderly fashion but also the manner in which a region of the genome is folded in a particular cell can determine the activity of the genes in that region [2]. The DNA of human and also most of the eukaryotic cells, is tightly bound with specialised proteins called histones, which tightly pack the DNA and regulate its activity. There are five major types of histone proteins which are rich in positively charged basic amino acids and therefore readily interact with the negatively charged phosphate groups in DNA. Nuclear DNA associated with histone proteins is called chromatin. Large organised molecules of chromatin are called chromosomes. The chromosomes are not visible in non-dividing cells, even under electron microscope. During mitosis and meiosis (stages of cell division) the chromosomes condense and become visible even under a light microscope[3].

1.4 The Cell Cycle

The cell cycle starts with the cell preparing for the division by synthesising the necessary components for DNA replication. Cell cycle contains various phases –G_1,S (DNA synthesis), G_2, and M (mitosis). Two check points exist, one between G_1 and S and one between G_2 and M as shown in Figure 1.1.

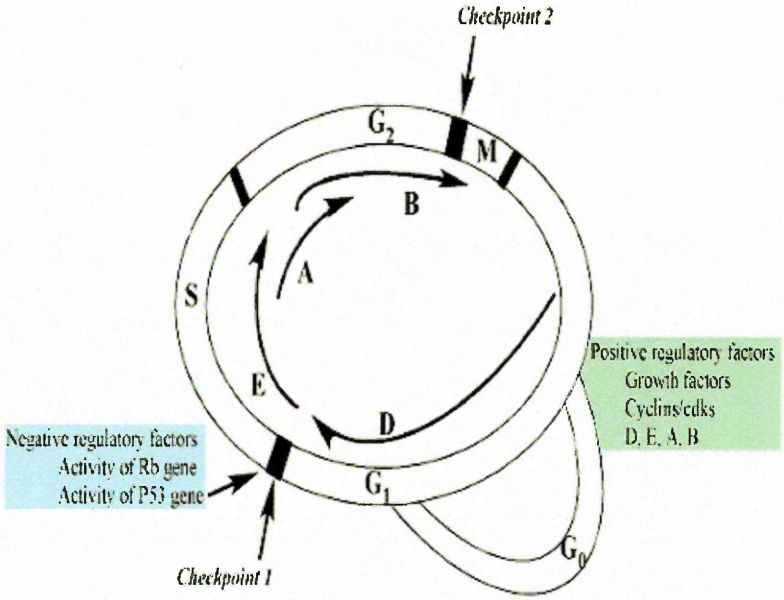


Figure 1.1 adapted from Pharmacology by Rang [4]

Growth factors constitute the positive force that stimulates the cell to start on the cycle. These growth factors are a series of cyclins and cyclin-dependent kinases (cdks). The action of these cyclin/cdks is controlled by various negative factors. They include proteins that bind to the cdks and inhibit their action. Induction of these proteins is by various genes, for example the *p53* gene and retinoblastoma gene (Rb) which are termed as 'superbrakes' on the cell cycle. In case of DNA damage, these inhibitors stop the cell cycle at checkpoint 1 as shown in Figure 1, allowing for repair [4].

1.5 Chemistry of DNA

DNA is constructed from four main nucleotide building blocks. These building blocks contain, a phosphate group linked to a five-carbon-atom sugar group, which in turn is linked to an aromatic molecule that can be either a purine (double ringed) or a pyrimidine (single ringed). DNA contains the sugar deoxyribose, hence the nucleotides are called deoxyribonucleotides. DNA is built up of two purine

containing nucleotides and two pyrimidine containing nucleotides. The purines of DNA are adenine and guanine. The pyrimidines are cytosine and thymine. Purine and pyrimidine bases containing sugar are known as nucleosides and are shown in figure 1.2.

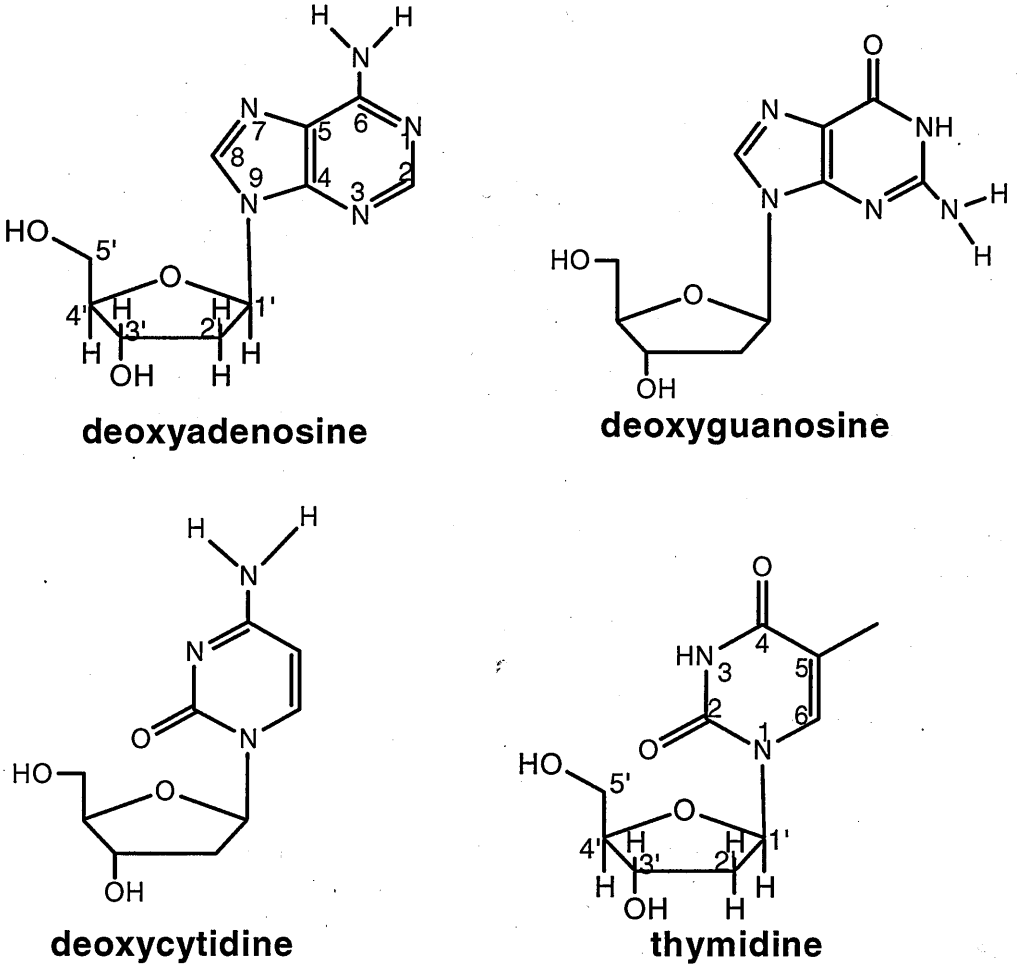


Figure 1.2: Structure and numbering of purine and pyrimidine nucleosides

Nucleotides of DNA are joined together by covalent bonds linking the phosphate group of one nucleotide to a hydroxyl group on the sugar of the adjacent nucleotide as shown in figure 1.3. These bonds are called phosphodiester linkages [5].

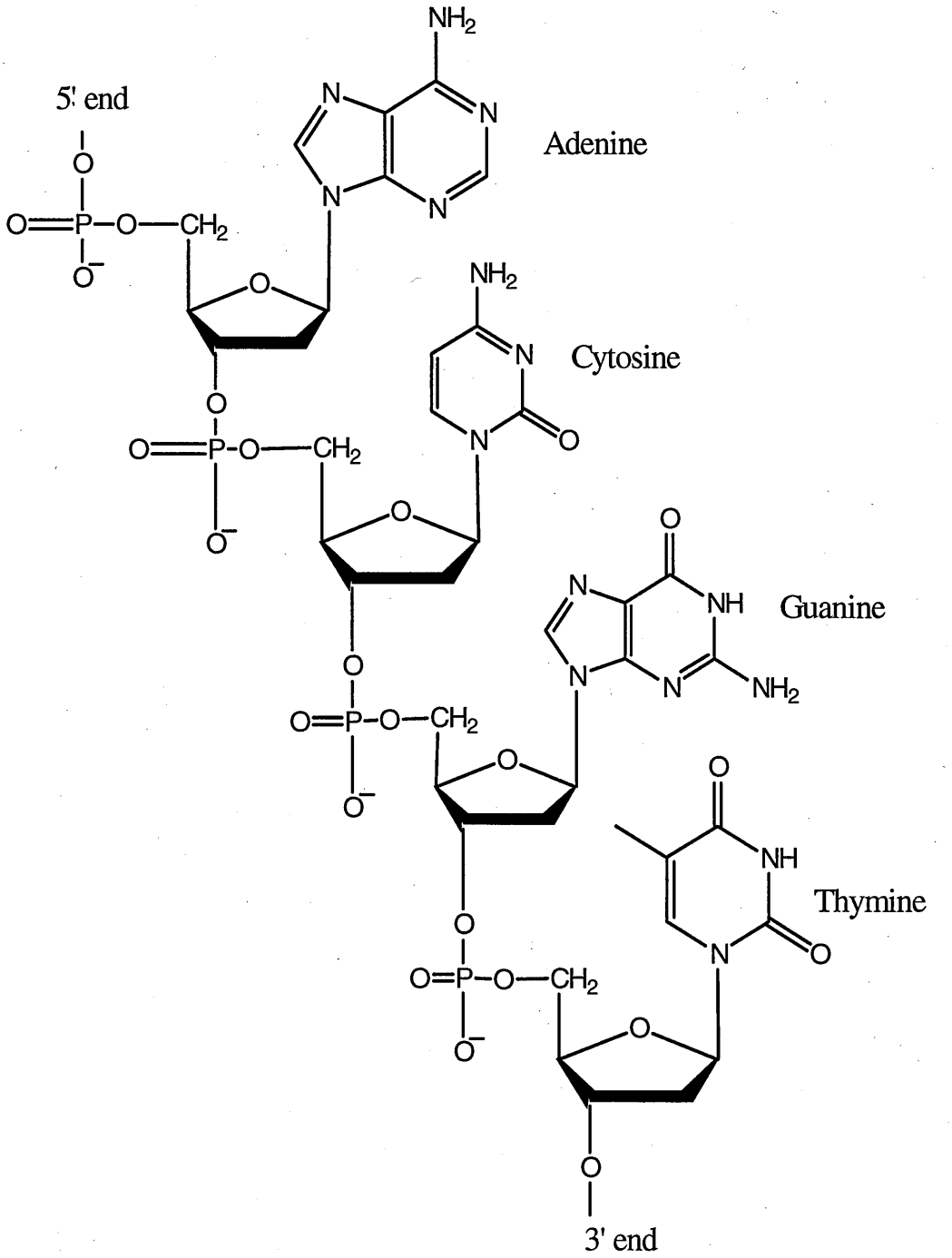


Figure 1.3 A portion of DNA polynucleotide chain, showing the 3'-5' phosphodiester linkages

DNA is an anti-parallel double helix held together by hydrogen bonds between pairs of bases on the opposing strands. The base pairing is restricted: the purine adenine only base-pairs with pyrimidine thymine, while the purine guanine base-pairs with pyrimidine cytosine[5]. The restrictions are due to both steric hindrance and

hydrogen bonding limitations. The helical nature of sugar-phosphate backbone does not allow two purines between the strands. Two pyrimidines fit between the strands but the bases would be too far away to form hydrogen bonds. Adenine donates 1H-bond and can accept another; thymine also can donate one and accept hydrogen in AT base pairing as shown in figure 1.4. Guanine can accept 1H-bond and can donate 2H when paired with cytosine, which can accept 2H-bonds and donates 1hydrogen in the GC base pairing.

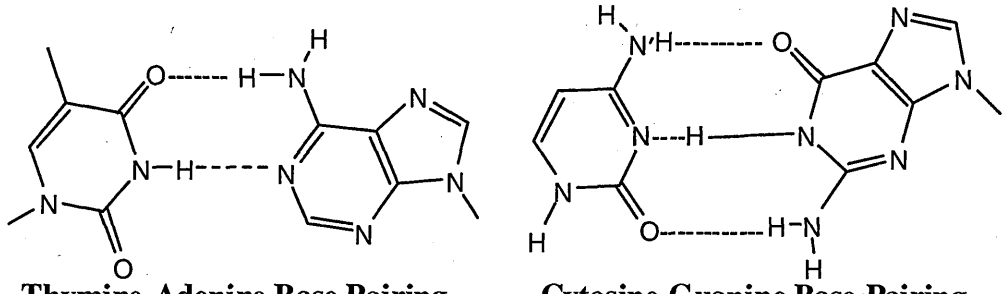


Figure 1.4 showing hydrogen bonds in AT and GC basepairs

The hydrogen bonds not only provide the specificity but also stability to the double helix structure [5].

1.6 DNA damage

DNA consists of various functional groups such as exocyclic amino groups, double bonds, keto and hydroxyl groups all of which are reactive. So, DNA is constantly damaged by both internal and external agents such as ultraviolet (UV) light, ionising radiations, chemicals and diet. Approximately 70% of cancers in western populations can be attributed to diet and lifestyle [6].

1.7 Damage to DNA by exogenous agents

1.7.1 Chemical Carcinogens

DNA is constantly damaged from exogenous agents both chemical and physical, which are commonly termed as environmental carcinogens. For both, the chemical reaction giving rise to DNA damage can be characterised as an electrophilic attack upon a tissue nucleophile [7]. Chemical attack on guanine is the most significant type. The chemical changes induced to guanine are known to interfere with base-pair recognition during replication. Environmental carcinogenesis was reported in as early as 1775 and involved tumour induction in chimney sweeps exposed to polycyclic hydrocarbons in soot [6]. Other chemicals such as dimethylnitrosamine, which was used as a specialised industrial solvent in the 1930s, was implicated in liver damage in exposed workers [8].

1.7.2 Diet

The accidental discovery of dimethylnitrosamine as a carcinogen was a major breakthrough. This discovery was interesting because it not only led to the detection of an industrial carcinogen but also to a new class of carcinogen, N-nitrosamines. Subsequently, the N-nitrosamines were identified in a number of consumer items from beer, to tobacco smoke and cosmetics [9].

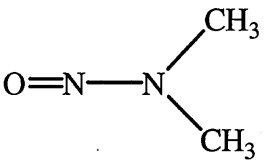


fig 1.5 N,N-dimethylnitrosamine

Nitrosamines were also found to be formed in the acid environment of the stomach with primary and secondary amine ingestion which are found in high levels in fish, and of sodium nitrite, which is found in salted fish as a preservative. The high incidence of gastric cancer in Japan and Iceland was linked to their diet which has salt-preserved fish as a major constituent. Epidemiological data suggests that red and processed meat intake also increase the risks of colorectal cancer. This might be due to the formation of heterocyclic amines formed while cooking the meat [10-13].

1.7.3 Physical Carcinogens

Ionising radiations and ultraviolet radiation (UV) can be considered as physical carcinogens. Although they differ in their chemical reactions both classes of physical carcinogens produce DNA damage which, as with the chemical carcinogens, can lead to mutations. DNA can be directly damaged with ionising radiations by causing single and double strand breaks in the helix and can also induce indirect damage by radiolysis of water to produce free radicals[14]. UV radiation has less energy when compared to IR and cannot produce ions. UV radiation can be absorbed by the DNA bases and is sufficient to induce chemical reactions. The most common of these reactions are between two thymidines in the DNA helix and results in the formation of cyclobutane-linked thymine dimers as shown in Figure 1.5 [15].

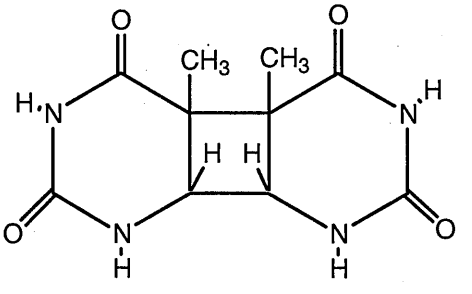


Figure 1.6 Cyclobutane linked dimer formation in adjacent thymidines

UV could also damage purine bases that might lead to potent toxic and mutagenic lesions [16-19]. Formamidopyrimidine derivates have been identified among other purine photoproducts [20]. These derivatives may result in the obstruction of DNA polymerase and if not repaired can give rise to mutations.

1.7.4 DNA damage by free radicals

Inside the cell a small fraction of oxygen is converted into superoxide as a by-product during the cellular metabolism of oxygen to water inside the mitochondria[21]. This superoxide may be converted into various reactive oxygen species (ROS) like hydroxyl radicals ($^{\cdot}OH$), H_2O_2 etc via various chemical and biochemical processes. Most of the cells possess defence mechanisms against the potential harmful effects of ROS. These defence mechanisms include catalase which converts H_2O_2 into water and oxygen and superoxide dismutase which converts its substrate, superoxide into oxygen and hydrogen peroxide [22]. The majority of DNA lesions induced by oxidative metabolism are single, but multiple lesions known as tandem lesions may appear on adjacent nucleotides [23].

Even though DNA is chemically damaged by various agents the damage of DNA itself is not a mutagenic event. Replication of DNA and cell division is necessary to convert the damage to an inheritable change in the DNA. In addition to chemical damage to DNA. spontaneous mutations can occur directly as a consequence of errors in replication [15]. Spontaneous DNA damage is a frequent event but as approximately 97% of DNA is non-coding in the human genome many base changes do not give rise to deleterious mutations [15].

1.8 DNA Repair

As discussed above, DNA is constantly exposed to chemical damage, both endogenous and exogenous, throughout evolutionary time. To check the genome for damage and/or repair, genes such as p53 have evolved. Other genes exist whose function is to repair errors introduced during the process of replication [15]. Mechanisms of repair differ according to the type of DNA damage. Thymine dimers are formed as a consequence of UV radiation. In this case, the whole stretch of DNA is removed and re-synthesised using the opposing strand as a template. Alkylated bases such as O^6-methylguanine can be directly removed without breaking the phosphate backbone. Single strand breaks can be directly repaired [15, 24]. DNA double strand breaks are the only type of damage that cannot be repaired. In this case because both the strands are damaged, the cell has no unmodified template that can provide the information necessary to repair the damaged strands. This type of damage could lead to cell death depending on the site of double strand break or, this could be of significance to the carcinogenic process, chromosome breakage and recombination with resulting activation or inactivation of crucial genes[15]. Defects in these repair processes play a vital role in the carcinogenesis by increasing the rate of mutations. Several genetically inherited diseases which give rise to cancers have their origin in defects of DNA repair capacity. These include ataxia-telangiectasia, in which cells are sensitive to X-rays [25] and Xeroderma pigmentosum which is sensitive to UV [26]. BRCA1 is the gene essential for repair in response to DNA damage in breast cancer [27]. One of the most frequently mutated genes in human solid tumours is p53 which has been called "the guardian of the genome". This gene not only has the functions of monitoring the integrity of genome but also has the capacity to delay

replication until the repair has been completed or if the damage is extensive it induces a series of events leading to programmed cell death by a process called apoptosis [28].

1.9 Cancer Biology:

Cancer is defined as a disease in which there is an uncontrolled multiplication and spread of the body's own cells. Incidence of human cancers increases with age. Cancer is one of the major causes of death in the developed nations. One in five people in Europe and North America die of cancer. Mutations in the genes have two distinctive consequences:

(a) Inappropriate expression or activation of genes

(b) Functional inactivation of the genes or its protein product

According to American Cancer Society two types of genes play a role in the development of cancer: Oncogenes and Tumour Suppressor genes. Oncogenes are mutated form of genes that cause normal cells to grow out of control and cause cancer. Tumour suppressor genes are normal genes that regulate cell division, repair DNA mismatches and bring programmed cell death (apoptosis). When tumour suppressor genes are mutated, cells can grow out of control leading to cancer. Oncogenes are involved in signalling pathways which stimulate proliferation and the tumour suppressor genes code for proteins which normally act as checkpoints to proliferation or cell death. The following five major pathways must be activated or inactivated for the cell to become malignant.

- Development of independence in growth stimulatory signals
- Development of refractory state to growth inhibitory signals
- Development of resistance to apotosis
- Development of an infinitive proliferative capacity

- Development of angiogenic potential (capacity to form new blood vessels and capillaries) [15].

1.10 Skin Cancer

Continuous exposure to UV radiation damages DNA of the exposed skin and might lead to cancer[15]. In the Northwest Europe the incidence of skin cancer is almost equal to that of lung or breast cancer, but in the U.S the skin cancer is the most common type[29]. Skin cancer could be classified into three main types: the most common type basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and cutaneous malignant melanoma (CMM) [29]. CMM (arising from melanocytes) is the cause of majority of the deaths from skin cancer though it is less common than BCC or SCC (arising from keratinocytes). These two are less aggressive and can be cured with relatively high success but if neglected, can become invasive. Skin cancers can be identified early in their development because they occur on the body surface which greatly enhances to the success of their therapy[30].

1.11 Cancer treatment:

The best form of treatment is prevention. Changes in lifestyle such as smoking control and dietary habits are necessary. Detecting the cancer early is also important. Treatment of cancer is generally based on the removal and/or killing of the tumour cells while minimising the side effects to normal cells.

Three main approaches are followed to treat the established cancer: surgical excision, irradiation and chemotherapy. The role of these approaches depends upon the type of tumour and the stage of its development. Chemotherapy is used as a main method for only certain types of cancers [4]. But it is used as an adjunct to

surgery or irradiation for many types of tumours. Many other approaches such as immunotherapy, angiogenesis inhibitors, gene therapy, and use of biological response modifiers (e.g. interferons, haemopoietic growth factors, etc) are under extensive investigation. Chemotherapy poses a problem because cancerous cells and normal cells are very similar in many respects and it is difficult to find general, exploitable, biochemical differences between them. The major difficulty in the use of cancer chemotherapy is that a tumour is usually far advanced before it is even diagnosed [4]

1.12 Drugs used for cancer chemotherapy

Most anticancer drugs act in S phase, the phase of cell cycle in which DNA synthesis occurs. Some act in M phase, where chromosomes are condensed and nucleus and cytoplasm divide. But some have complex actions in the cycle. Cell cycle progression is regulated by positive and negative forces[31].

The main anticancer drugs could be categorised as follows:

- Cytotoxic drugs:

As the name suggests these drugs induce toxicity to the cancerous cells by different mechanisms as shown in figure 1.6 leading to cell death. These drugs can be further categorised into

-alkylating agents and related compounds: these agents form covalent bonds with DNA and interfere in its replication

-antimetabolites: these agents block the metabolic pathways involved in DNA synthesis. Antimetabolites are the structural analogues of naturally occurring compounds that interfere with the synthesis of nucleic acids. They can be further classified into antifolates, purine analogues and pyrimidine antimetabolites.

-cytotoxic antibiotics: agents of microbial origin that prevent mammalian cell division

-plant derivatives: most of these affect microtubule function

- Hormones:

These agents mostly comprise of steroids such as glucocorticoids, oestrogens and androgens. These drugs mostly suppress hormone secretion or antagonise hormone action.

- Miscellaneous Agents:

Drugs that do not fit into the above categories.

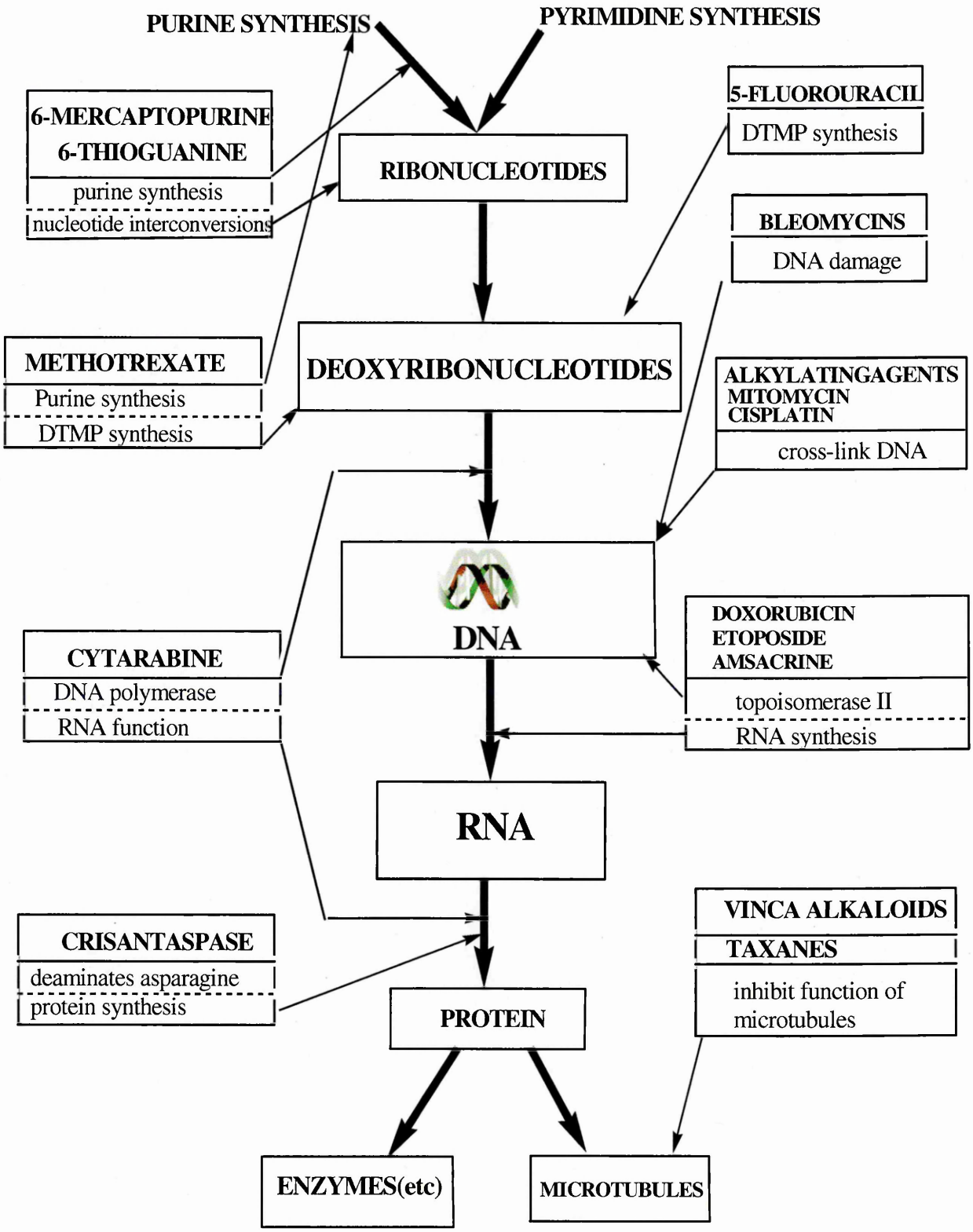


figure 1.7'Action of cytotoxic agents on the dividing cells'. Adapted from Pharmacology b Rang [4]

DTMP= 2' deoxythymidilate

1.13 Carcinogenic Cancer Chemotherapeutic Agents

Many cancer chemotherapeutic agents show their pharmacological activity by causing DNA damage. Patients surviving the therapy with these agents are at high risk of iatrogenic cancer [32]. Even the radiotherapy has the same implications but the localised nature in comparison to systemic therapy with chemotherapeutic agents, limits the overall risk of secondary cancer. Alkylating agents such as cyclophosphamide bring out their pharmacological action by reacting with DNA. This action is similar to the carcinogens as discussed previously. Other class of compounds include antibiotics such as, doxorubicin that interact non-covalently with DNA and induces DNA damage by free radical mechanism [15]. So, paradoxically, most of the available cancer chemotherapeutic agents are carcinogenic themselves.

1.14 Nucleosides and analogues as drugs

Uptake of nucleosides into the cells is mediated by nucleoside transporters (NTs) [33]. Specialised cells such as enterocytes, bone marrow cells and certain brain cells lack de novo synthesis pathways [34]. These cells salvage nucleosides from the extracellular milieu for the production of nucleotides to use in RNA and DNA synthesis. Nucleotides play an important role as intermediates for many essential cellular biosynthetic pathways. Other key roles of nucleotides include neurotransmission [33] and regulation of cardiovascular activity [35] and as signalling molecules [36]. Nucleoside kinases present inside the cells mediate the phosphorylation of nucleosides to nucleotides [37]. Apart from these functions nucleosides are also used as drugs for treatment of some conditions for example adenocard (unmodified adenosine) is used as a drug to slow tachycardia (a condition where heart rate increases). Also naturally occurring nucleosides and nucleotides

have been chemically modified for more than 50 years and various analogues have been synthesised to exploit mostly their antiviral and anticancer properties[38-41]. The cellular uptake of anticancer and antiviral nucleoside drugs is similar to that of natural nucleosides i.e. mediated by nucleoside transporters (NTs)[33]. The modifications are of two types:

- sugar modified nucleosides
- base modified nucleosides

The sugar is usually modified at 2' or/and 3' or/and 5' positions [42-44]. Sugar modified nucleosides are mainly used as antiviral and anticancer agents for example Zidovudine (AZT) which is used in the treatment of AIDS is modified by insertion of an azide group at the 3' position in the sugar of thymidine [45-47]. Usually base modified nucleosides are synthesised by replacing oxygen or hydrogen atom of uracil, thymine or guanine with a suitable leaving group and replaced by the desired atom or group[48] like for example 5-fluorouracil (5-FU). In this drug the hydrogen atom at the fifth position of uracil was substituted by fluorine atom. The cellular toxicity was attributed to the presence of fluorine. 5-FU is metabolised in a similar manner to uracil. This was attributed to the fact that atomic radius of fluorine is similar to that of hydrogen (1.35A compared to 1.2A) and that the bond between the carbon and fluorine is extremely stable. 5-Fluoro-2'-deoxyuridine (FdUrd), the corresponding deoxyribonucleoside derivative of 5-FU also has chemotherapeutic activity. 5-FU and FdUrd, along with other agents are now the most widely used drugs in the treatment of advanced colorectal cancer as well as many other cancers including breast, head/neck carcinomas [49-51].

1.15 Sulphur modified nucleosides

Sulphur modified nucleosides are an important class of modified nucleoside analogues. These sulphur modified analogues can be classified into two kinds;

- sulphur modification in the sugar
- sulphur modification in the base

It was reported that the replacement of oxygen with sulphur in the sugar of thymidine and deoxycytidine gave nucleosides antiviral and antitumour properties [52, 53]. Base modified analogues are also used for therapeutic activities, for example, 6-mercaptopurine has been used since the 1950's for the treatment of acute leukemia [54] and azothioprine is used as an immunosuppressant.

Apart from their therapeutic activities a number of other applications have been reported for thiopyrimidine and thiopurine nucleosides and nucleotides. For example thio derivatives of adenosine are used as affinity labels for pyruvate kinase [55, 56] and photo-affinity label of DNA polymerase I Klenow fragment [57]. Thioadenosine and its phosphate derivatives were also used as intermediates in the synthesis of various photo-reactive nucleosidic, nucleotidic and oligonucleotidic probes. Other thioadenosine derivatives such as 8-[(4-azidophenacyl)thio]adenosine 5'-triphosphate were used as a photo cross-linking agents[58]. 4-thiouridine is present as a natural constituent of t-RNA and is used as an intrinsic photo-label in the study of nucleic acids [59, 60]. Photo cross-linking ability of 4-thiopyrimidine and 6-thiopurine nucleosides has been used to study the three-dimensional interactions between RNA-RNA or RNA-proteins. Thiopurines and thiopyrimidines are activated selectively by UVA light (330-350nm) and the specific contacts were mapped using photo cross-linking [60-64]. Sulphur modification of thymidine on the 5-methyl group could be achieved by 5-chloro derivative and the 5-thiomethyl derivatives prepared from this

intermediates have efficient anti-herpes activity [65]. Apart from these uses, sulphur modified nucleosides are also used as tools in studies of protein or nucleic acid structure and functions. The thionucleobases are used as intrinsic photolabels to probe the structure in solution of folded RNA molecules and to identify contacts within nucleic acids and between nucleic acids and proteins, in complex nucleoprotein assemblies. The thiobases absorb light at wavelengths longer than 320 nm and thus can be selectively photoactivated. The photoadducts formed are studied. The photocrosslinking potential of thionucleosides inserted in nucleic acid chains has been used to probe RNA-RNA contacts within the ribosome. These can also be used as anti-sense modulators of gene expression [48].

1.16 Thiopurines as drugs

The cytotoxic thiopurine agents 6-mercaptopurine, 6-thioguanine and azathioprine are used as prodrugs and must be metabolised before they can interfere with the synthesis of DNA [66]. The presence of a reactive thiol group was attributed to their cytotoxic and immunosuppressive properties. The activity of thiopurines is via the incorporation into DNA as thioguanine nucleotides or inhibition of *de novo* purine synthesis by methyl thioguanine nucleotides [67]. 6-Mercaptopurine is widely used in primary treatment of childhood leukaemia and as an investigational agent for the treatment of Crohn's disease and the related gastrointestinal disorders. 6-Mercaptopurine is catabolised by xanthine oxidase to the inactive metabolite 6-thiouric acid. A second catabolic pathway involves thiol methylation by the enzyme thiopurine methyltransferase which converts 6-thioinosine monophosphate into 6-thioinosine monophosphate [68, 69].

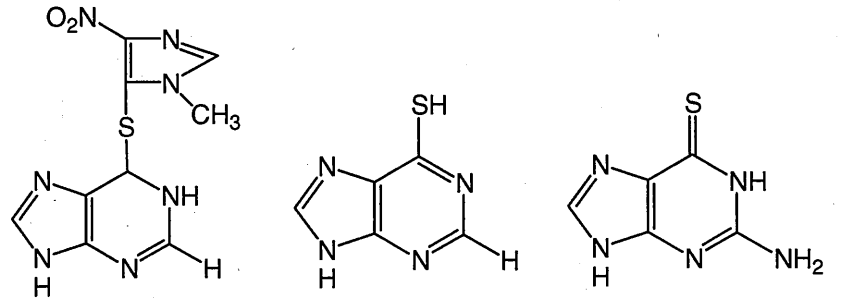


Fig 1.8 Chemical structures of thiopurines used for anti cancer therapy

Azathioprine molecule contains two main moieties as shown in figure 1.8, a mercaptopurine and imidazole derivative. It is metabolised to form mercaptopurine and methyl-4-nitro-5-imidazole derivatives are metabolised by suplhydryl (SH) and amino group (NH) containing compounds like cysteine [70, 71]. The first step in the nucleophilic attack of the COO(-) of cysteine on the C(5i) atom of the imidazole ring of azathioprine molecule [70, 71]. Azathioprine is used as an immuno suppressant along with other drugs for solid organ transplantation. This drug is also under investigation for the treatment of rheumatoid arthritis, vasculitis and dermatological disorders[72]. 6-thioguanine has been used as a therapeutic agent in the treatment of cancer since the early 1950s[54]. Similar to azathioprine and 6-mercaptopurine, 6-thioguanine also requires activation to 6-thiodeoxyguanosine 5'-triphosphate through metabolic pathways before it's incorporation into DNA[73, 74].

1.17 Thiopyrimidines as drugs

Unlike thiopurines not many thiopyrimides have been developed as drugs even though many derivatives have been synthesised for more than 50 years. Antiviral activity of 2-thiopyrimidine and its 5-halogenated derivatives has been previously reported[75]. Anticancer activity of these compounds has not been thoroughly

investigated. However incorporation of 4-thiothymidine (T^S) into DNA and the synergistic effect of UVA light in sensitising the human cancerous cells to a very low, non lethal UVA doses have been reported[67]. Cellular incorporation and photo chemical properties of 4-thio- 5-bromo-2'-deoxyuridine (S^4-BrdU) have also been reported [76].

Data available for most of the thiopyrimidines, for example T^S and S^4-BrdU suggests that they are not toxic to cells over a range of concentrations unlike thiopurines. Cellular incorporation reports have suggested that T^S was a substrate for thymidine kinase (TK) but not for thymidine phosphorylase (TP) and the incorporation of T^S into DNA is mediated by thymidine salvage pathway. It was also reported the T^S was not very toxic when incorporated into the cells. In another report Massey et al[67] have demonstrated T^S to be a weak mutagen when compared to other thiopurines. It replaces thymidine when incorporated into the DNA. They have also reported that T^S generally codes to direct the incorporation of adenine (A) during replication. These properties make T^S an ideal candidate for cellular incorporation and further manipulation.

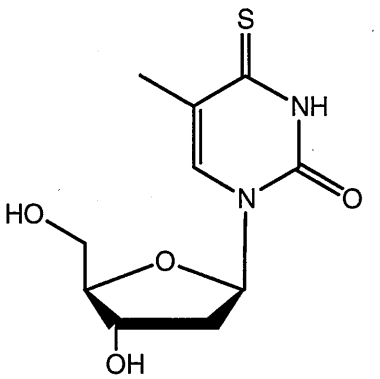


Figure 1.9: Thiopyrimidines:Structure of T^S

The substitution of oxygen by sulphur at position 4 as shown in Figure 1.8 shifts the absorption spectrum from 267nm to 335nm. This property makes it easier to identify

it in the presence of the other nucleosides which have a maximum absorption around 260nm [77]. Thiopyrimidine also sensitises cells when exposed to UVA light. Other properties such as cross-linking of DNA could also bring about cell killing.

1.18 Cross-linking

As mentioned in figure 1.6 the cytotoxic action of the alkylating agents such as mitomycin and cisplatin is due to the formation of interstrand cross-links within the DNA strands[4]. Apart from these alkylating agents many other possible anticancer drug candidates also exert their cytotoxic action by forming cross-links. These cross-links are non-specific leading to hazardous side effects. As mentioned earlier guanine is the major site of modifications for most of these drugs[78]. Moreover formation of mismatch is the major disadvantage with the other drugs if used for this purpose. The 'not very toxic', weak mutagenic nature and coding exclusively for adenine makes 4-thiothymidine an ideal candidate for cross-linking.

1.19 DNA-Protein cross-linking:

DNA and protein interactions are very important in the cellular processes from DNA replication to gene regulation [79]. These interactions are vital for the survival of a living organism. For example, as mentioned earlier, cells are under constant threat by DNA damaging agents like chemical and physical carcinogens. When the DNA damage occurs a number of biochemical signalling pathways are activated to repair the damage. The process of repair is carried out by the proteins which interact with the DNA [80]. Interestingly, purposeful cross-linking of proteins to DNA interrupts DNA metabolic processes such as replication, repair, recombination, transcription and chromatin remodelling. The effects of agents that cause DNA-protein cross-links

(DPCs) have been investigated[81-83]. DPCs physically block the progression of replication or transcription complexes and prevent access of proteins required either for synthesis along the template strand, for transcription, or for repair recognition and/or incision. By anchoring to the chromatin they may also affect all of these processes and prevent its remodelling [84].

Several methods have been developed for cross-linking of proteins to DNA but only a few such as photo cross-linking and chemical cross-linking were successful. The principle involved in the photo cross-linking technique is that an agent is irradiated with a suitable light source and the level of cross-linking is determined [85-88]. The agents used for photo cross-linking include azide derivatives, halogenated bases and thio-bases etc [89-92]. Non specificity of the reaction is the main disadvantage with photo-cross-linking[88].

Chemical cross-linking reactions are either nucleophilic or electrophilic substitutions but could be non specific because the DNA has many nucleophilic sites such as keto, exocyclic amino, double bonds and hydroxyl groups. Xu and others [88, 93] have reported a method for site specific and chemical cross-linking of oligodeoxynucleotides containing 6-methylsulphoxypurine with cysteine and peptides containing cysteine. In this method the author achieved the specificity of the reaction by creating an electrophilic site with a synthetic fragment of the DNA (pentamer). Creating an electrophilic site that is specific for an incoming nucleophile in the cellular DNA could be a tedious and laborious process and probably practically impossible to achieve. This is because, as discussed earlier DNA has many reactive sites. The post-synthetic approach [85] is another strategy in which a monomer containing a leaving group is incorporated into the oligomer which is then replaced by suitable nucleophiles to obtain the desired product. 1,2,4-Triazolo group was

introduced as a leaving group and manipulation was done by various thio-nucleophiles. This approach also has similar disadvantages like that of the chemical cross-linking method where synthesising DNA with a suitable leaving group and introducing into the cellular DNA might not be feasible.

1.20 Aims and approaches of the current work.

In this project combination of both the approaches would be followed. Two thio modified nucleosides were selected for the purpose of this work. The primary objective in selecting these nucleosides was that, they were proven to be not cytotoxic when incorporated into the cells. 4-Thiothymidine and 4-thio-5-bromodeoxyuridine were chosen. The fact that 4-thiothymidine and 4-thio-5-bromodeoxyuridine could be easily incorporated into the cellular DNA with little toxicity [67] and that the thio group could be used for further modifications [94] has prompted us to follow this approach. Previously the thio group of modified nucleoside was used as a tool in the areas of molecular biology and cancer research [95]. The thiol group in oligonucleotides was also used for the synthesis of other modified oligonucleotides by post synthetic modification [96]. So the thio group of 4-thiothymidine and 4-thio-5-bromodeoxyuridine could be used as site specific chemical cross-linker of the DNA. In order to achieve this, first the thiol is modified by a reasonably stable and good leaving group and then cross-linked by amino acids and peptides containing thiols. Subsequently, the modified nucleoside(s) would be incorporated into the DNA and the cross-linking with the DNA strand would be explored with the same amino acids and peptides containing thiols. Then a protein would be chosen to study the interaction between the DNA and protein.

CHAPTER 2

SYNTHESIS AND REACTIONS OF 4-THIOTHYMIDINE

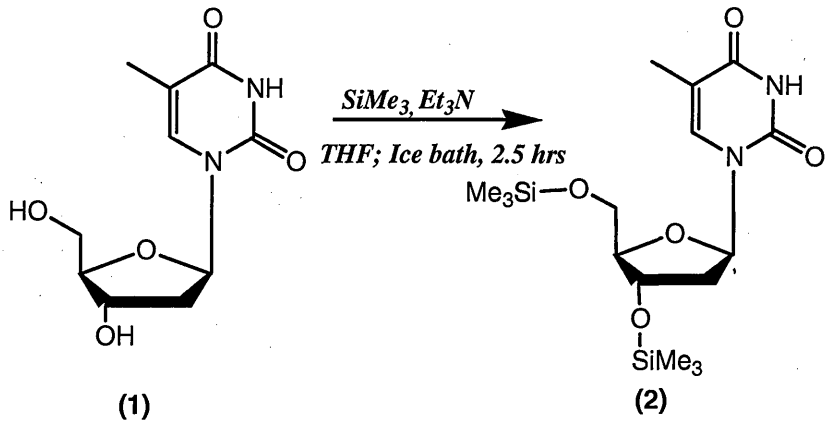
2 Synthesis and Reactions of 4-Thiothymidine

The synthesis of T^S was first reported by J.J. Fox et al in 1959 [97]. The synthetic procedure has been simplified and reported by various authors since then [98] [99]. Physically 4-thiothymidine is yellow coloured powder with chemical formula of $C_{10}H_{14}N_2O_4S$ and the formula weight is 258.30. It is structurally similar to the pyrimidine nucleoside thymidine except for the oxygen at C4, which has been replaced by sulphur. The reported protocol [98] was used for the synthesis which involves four steps as shown below.

2.1 Synthesis of 4-thiothymidine

Step-1

The first step involves protection of 3' and 5' OH groups of sugar with trimethylsilyl groups as shown in Scheme 1. Thymidine (**1**) was suspended in THF and reacted with trimethyl chlorosilane in the presence of triethyl amine to give 3`,5`-O-bis-(trimethyl silyl)-thymidine (**2**).

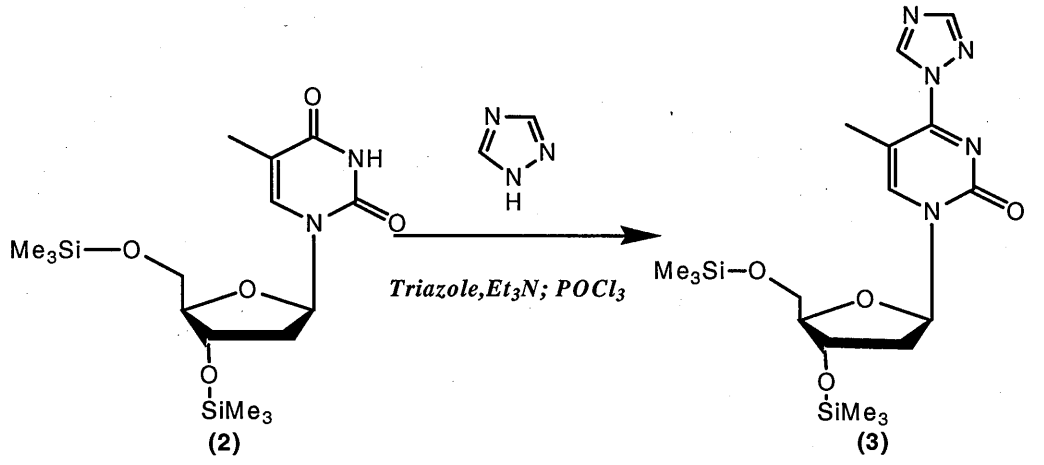


(1) (2)

Scheme 1 Protection of 3' and 5' OH of thymidine The OH groups are highly reactive. So 3' & 5' OH groups are protected with $SiMe_3$

Step-2

2 in acetonitrile was treated with 1,2,4-1H triazole in the presence of triethylamine and phosphorous oxychloride to give 3`,5`-O-bis-(trimethyl silyl)-4-trizolothymidine (**3**) as shown in Scheme 2.



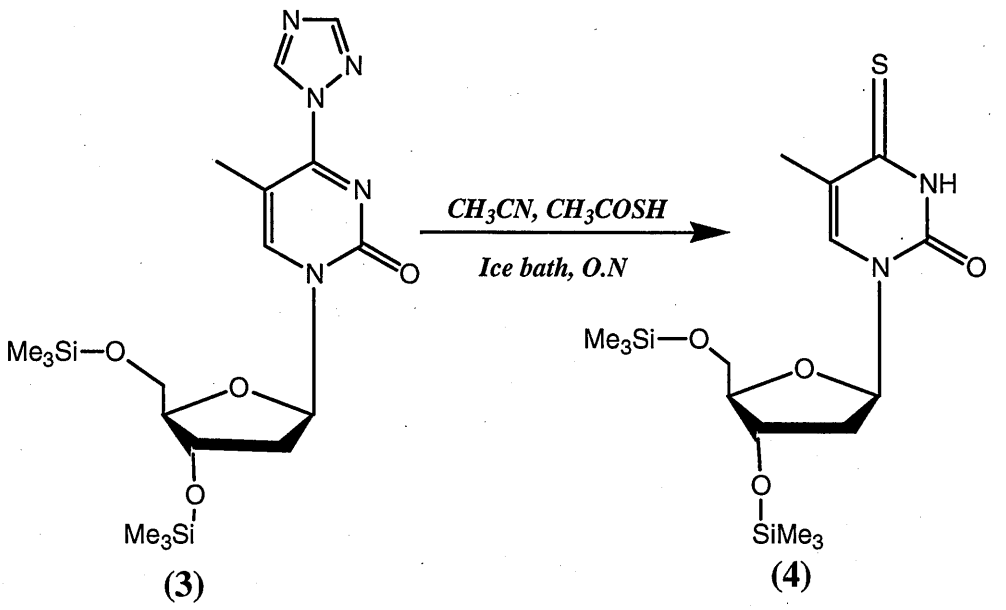
Scheme 2 Triazolation of C4 of thymidine

This reaction takes approximately 16 hours for completion

The key intermediate in this reaction is phosphoryl-*tris*-triazolide which is formed by the reaction of phosphorous oxychloride and 1,2,4-(1H)-triazole in the presence of triethylamine in acetonitrile. Triethyl amine is added in 2:1 molecular ratio in the beginning of the reaction and is not only used to neutralise the formed HCl but also to abstract a proton from 1-N of triazole. The phosphorous in the formed phosphoryl-*tris*-triazolide then reacts with the oxygen at C4 of thymidine to give 3`,5`-O-bis-(trimethylsilyl)-4-phosphoryl-*tris*-triazolothymidine. The lone pair of electrons on the nitrogen of triazole is then set up for attack at C4 to form 3`,5`-O-bis-(trimethylsilyl)-triazolothymidine (**3**) [100-102]

Step-3

3 in acetonitrile was treated with thiolacetic acid to give 3`,5`-O-bis–(trimethylsilyl)-2`-deoxy-4-thiothymidine (**4**).

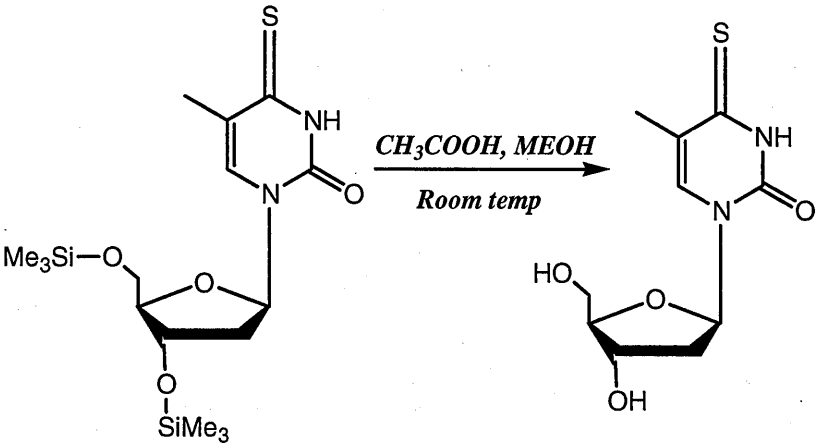


Scheme 3 Thiol group formation at C4 Triazolo group is replaced with sulphur. At this stage 3' & 5' OH groups are still protected

Protonation of the triazolo group affords a thioacetate ion which can then attack at C-4. Subsequent loss of the acetyl group probably occurs because the thio-keto function is more stable than 4-S-acetyl derivative.

Step-4

The trimethylsilyl groups are unstable in the presence of acid and 4-thiothymidine **(5)** is produced in high yield (more than 90%) by treatment of **4** with acetic acid in methanol.



(4) **(5)**

Scheme 4 Addition of acetic acid deprotects 3' & 5' hydroxyl groups

Miah *et al* [99] have simplified the procedure and in the final step performed silica gel column chromatography to obtain the pure compound. This method has been further simplified by us without having to perform column chromatography for purification and the compound obtained was highly pure when checked on HPLC (see chapter 5-materials and methods section for detailed procedure.

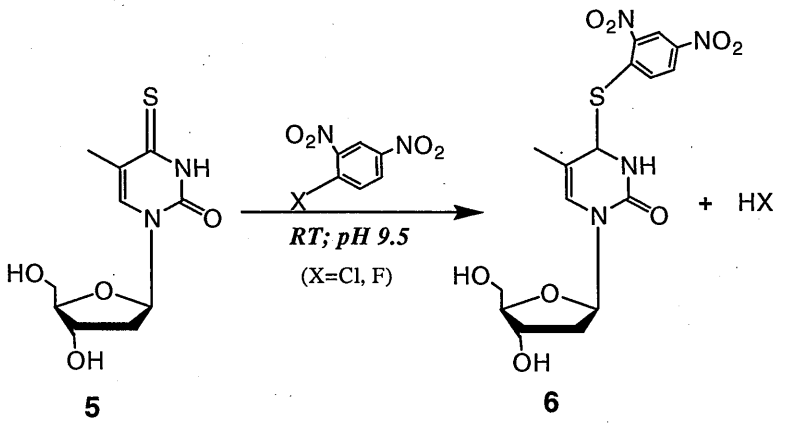
2.2 Modification of 4-thiothymidine

2,4-Dinitrophenyl (DNP) is a stable and good activating moiety for leaving groups allowing heteroatoms to be replaced by nucleophiles [103]. Syntheses of 2,4-dinitrophenyl-containing thiopurines as phosphoramidites such as 6-(2,4-dinitrophenyl)-thiopurine and 2-amino-6-(2,4-dinitrophenyl)-thiopurine and their

incorporation into oligonucleotides have been reported [103-105]. However this approach has not been reported for pyrimidines such as thiothymidine and this was chosen as the next stage.

2.3 Introduction of 2,4-dinitrophenyl group onto 4-thiothymidine

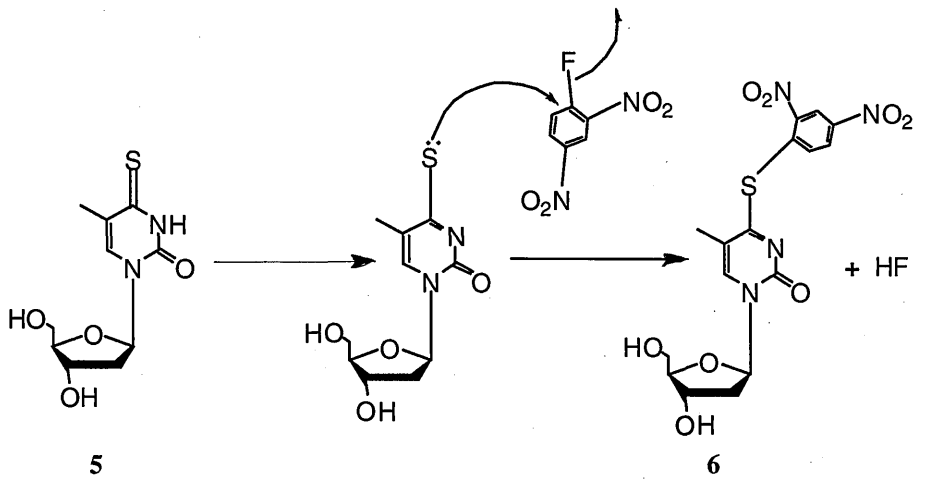
5 was treated with 2,4-dinitrohalobenzenes (1-Chloro-2,4-dinitrobenzene and 2,4-fluorodinitrobenzene (Sanger reagent)). The reaction was carried out at room temperature at pH 9.5 and monitored by HPLC at different time intervals.



5 **6**

Scheme 5 formation of T^{sdnp} from the reaction of T^{S} and halodinitrobenzenes

Possible reaction mechanism



5 **6**

Scheme 6 Reaction of (5) with FDNB

In the following reactions the molar ratio of T^S to reagents was 1:1. A typical HPLC profile was shown in figure 2.1.

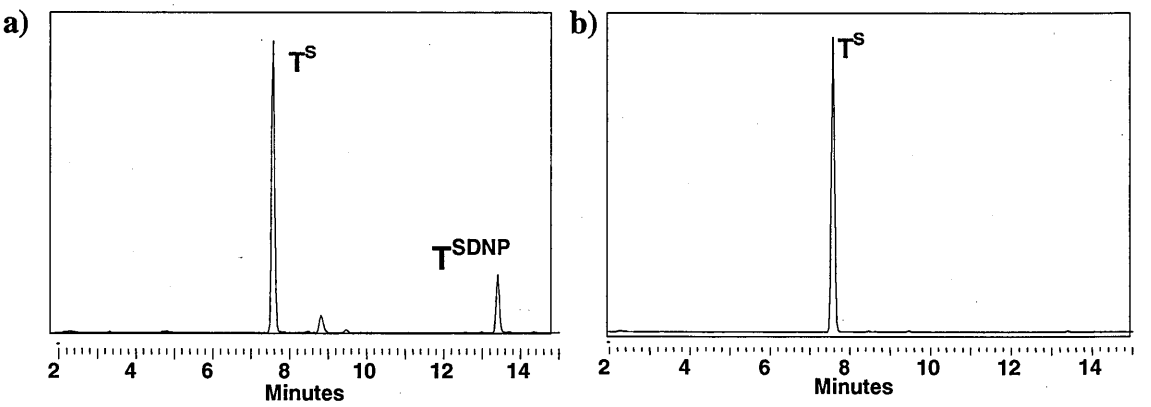


Figure 2.1: (a) HPLC trace of reaction of T^S with FDNB and b) HPLC trace of reaction of T^S with ClDNB, the reagent peaks were not shown.

The product (T^{SDNP}) is formed at a much quicker rate when FDNB was used.

After 1hour a new peak of T^{SDNP}, putatively, was observed at the retention time 13.5 minutes in figure *2.1 (a)* but not in figure *2.1 (b).* Peak at retention time 7.4 minutes is that of starting material **5**. The reaction was left at room temperature and monitored by HPLC after 24 hours (profile not shown) and 48 hours (shown in figure 2.2).

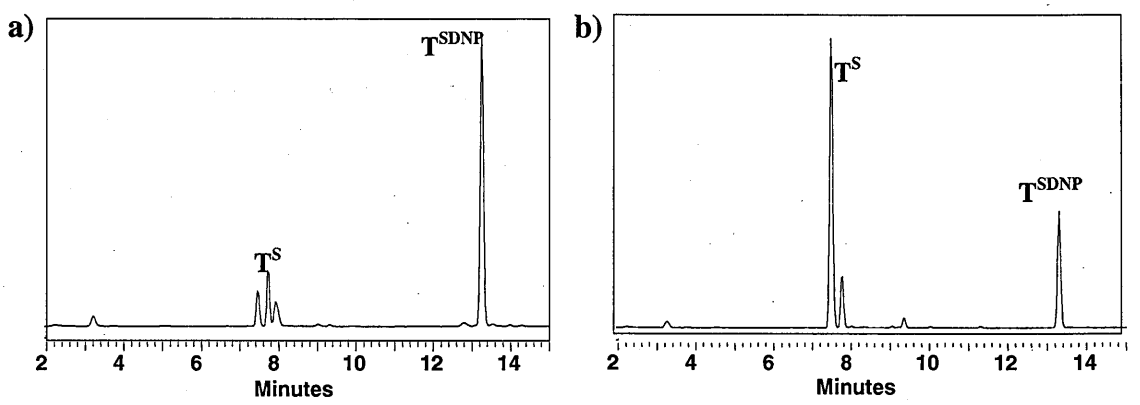


Figure 2.2: HPLC trace of reaction of T^S with

(a) FDNB after 48 hours. (b) ClDNB after 48 hours

Most of the starting material was converted to product when FDNB was used as a reagent after 48 hours

After 48 hours most of **5** was converted into a new peak in Figure *2.2 (a)*. In Figure *2.2(b)* **5** was converted into a new peak with the same retention time and UV spectrum. So the same product was observed with both reagents. The new peak has the UV spectrum as shown in figure 2.3

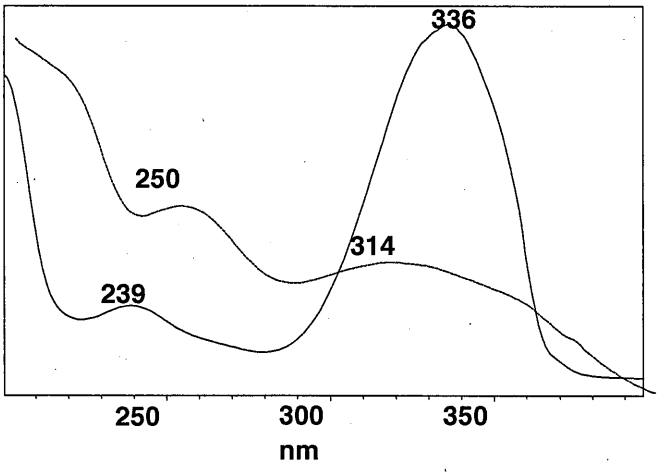


Figure 2.3 UV spectrum of $T^S(\lambda_{max}$ 336) and $T^{SDNP}(\lambda_{max}$ 314nm)

T^{SDNP} can be easily separated form T^s by UV difference

The reaction was thiol specific as no reaction with **1** was observed under the same conditions (Figure not shown). The same product was observed at a faster rate when 2, 4-dinitrofluorobenzene (FDNB or Sanger reagent) was used (explain why). FDNB was therefore used in subsequent reactions.

The reaction was explored further and a more rapid and greater increase in the formation of **6** was observed when excess of phosphate buffer was added to the reaction mixture. This could be due to the neutralisation of HF produced during the reaction.

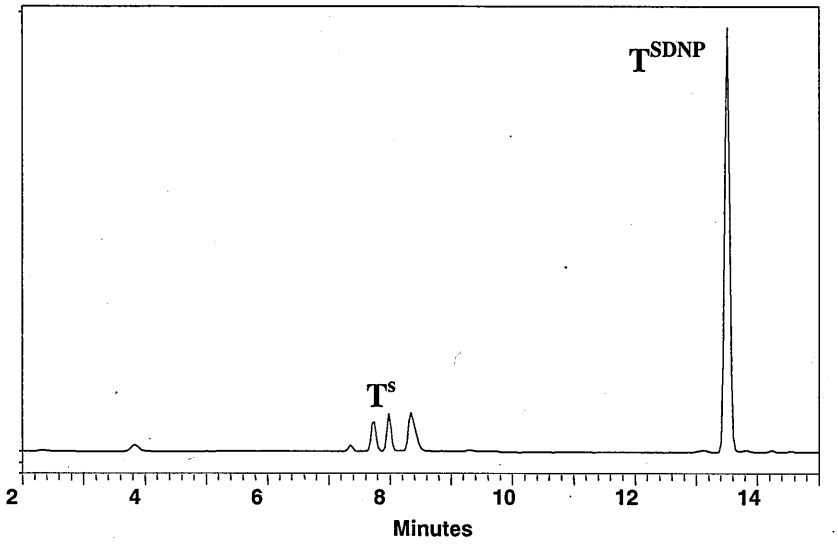


Figure 2.4: HPLC trace of addition of excess phosphate.

Most of the starting material (T^s) was converted to product (T^{SDNP}) after O.N

6 has a longer retention time and can be well separated from the starting material 4-thiothymidine and from thymidine. A typical HPLC separation profile is shown in Figure 2.5.

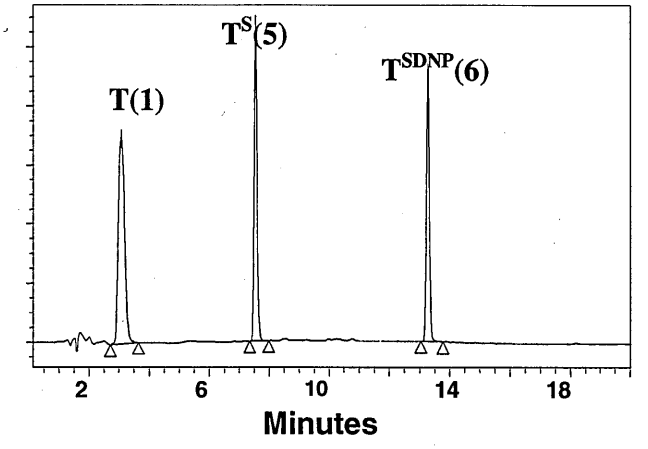


Figure 2.5 T^{SDNP} can be well separated from starting material (T^s) and Thymidine (T^0)

As shown in the figure 2.5, **6** could be easily separated and can be isolated from compounds (**1**) and (**5**) and the isolated peak of **6** shows a high degree of purity on HPLC as seen in figure *2.6*.

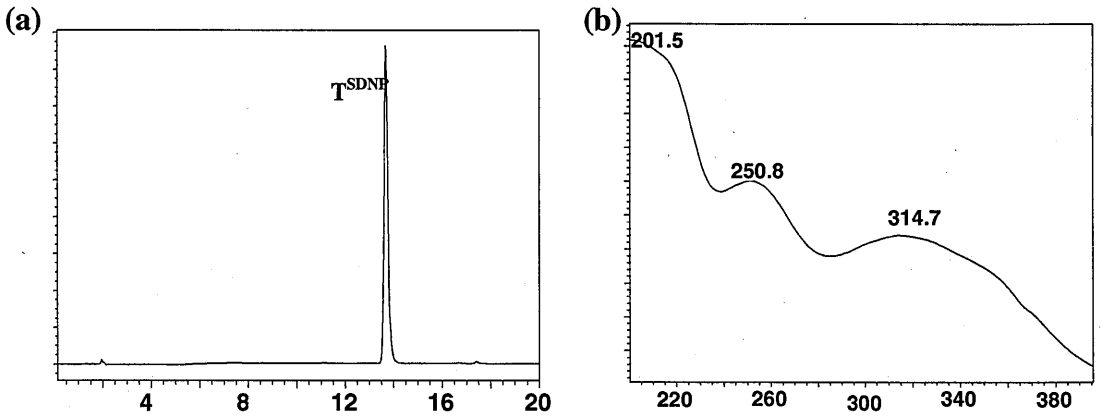


Figure 2.6 (a) Isolated peak of T^{SDNP} (b) UV of T^{SDNP}

Phenomenon Synergi Max 250 X 10 mm column was used for the isolation of T^{SDNP}

Mass spectral data of the new peak was obtained and it was tentatively assigned as S^4-2,4- dinitrophenylthiothymidine or **6**. The observed fragments were 424 M^+, 423 $(M^+-H)^+$ and 425 $(M^+H)^+$.

A Similar kind of approach has been reported by Xu [94] with thiopurine nucleosides. It was reported that unwanted 6-thiopurine analogues were formed through hydrolysis when 6-(2,4-dinitrophenyl)-thiopurine was used. Xu attributed this to the fact that 6-(2,4-dinitrophenyl)-thiopurine has two potential sites of nucleophilic attack i.e. purine C-6 resulting in the displacement of the dinitrophenylthio group and the C-1 carbon of the dinitrophenyl group resulting in displacement of the 6-thiopurine group. Both pathways involve displacement of good leaving groups. To overcome the unwanted formation of thiopurine during nucleophilic substitutions, Xu replaced the strongly

electron-withdrawing dinitrophenyl by less electron-withdrawing alkyl groups. However, this reduced the good leaving capability of dinitrophenylthio significantly. Nyilas and Chattopadhyaya [106] have introduced mono-nitrophenyl on the C4 oxygen of 2'-O-methyl-cytidine and other derivatives. Also Miah et al [99] have reported that nucleosides containing mono-nitrophenyl groups on oxygen were stable and could be readily replaced. So instead of an alkyl group a mono nitro reagent was tried in this experiment to reduce the strong electron-withdrawing effect of dinitro. It was also presumed that the good leaving capability of nitrophenylthio group is retained.

So, reaction with 1-fluoro-2-nitrobenzene and 1-fluoro-4-nitrobenzene under the same reaction conditions were monitored for 48 hours. No product was observed and a typical HPLC profile was shown in Figure 2.7 for overnight reaction.

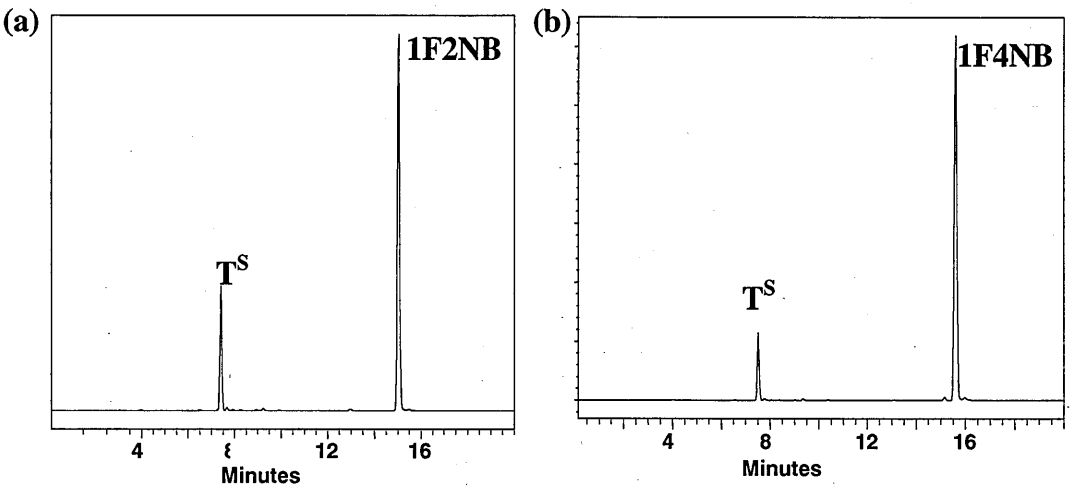


Figure 2.7 HPLC traces for overnight profile of T^S reaction with (a) 1F2NB and (b) 1F4NB

No new peaks were observed after leaving for overnight.

Since no new peaks were observed, both fluoro-2-nitrobenzene and fluoro-4-nitrobenzene were deemed unsuitable reagents and the product with FDNB i.e. **6** would be used for further manipulations.

Knowledge of the hydrolytic stability of **6** would provide useful information for its further manipulation with various nucleophiles. So the stability of **6** was studied.

2.4 Stability studies of T^{SDNP}:

In the aqueous solution T^{SDNP} was slowly hydrolysed to two new peaks. Highlights of the studies were, when pH of the solution was kept between 4 and 9 at room temperature, less than 5% of T^{SDNP} was hydrolysed after 24 hours. The compound seemed to be very stable when left in pH 6-8 for 24 hours. At pH 7 and 8, the hydrolysis was still very slow and over 60% of T^{SDNP} remains unchanged even after 9 days. At pH 10 a quarter of T^{SDNP} was hydrolysed in a day as shown in figure *2.8*.

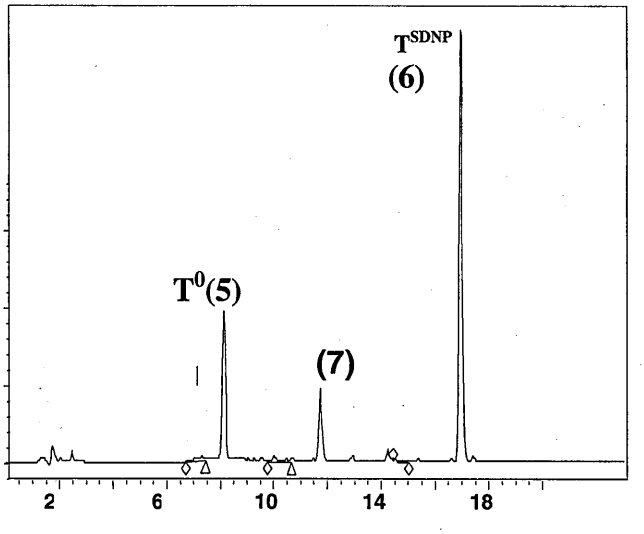


Figure 2.8: 1M concentration of phosphate buffer pH 10 was added to T^{SDNP} and the solution was injected on HPLC after 24hours

The complete studies are represented graphically in Figure 2.9.

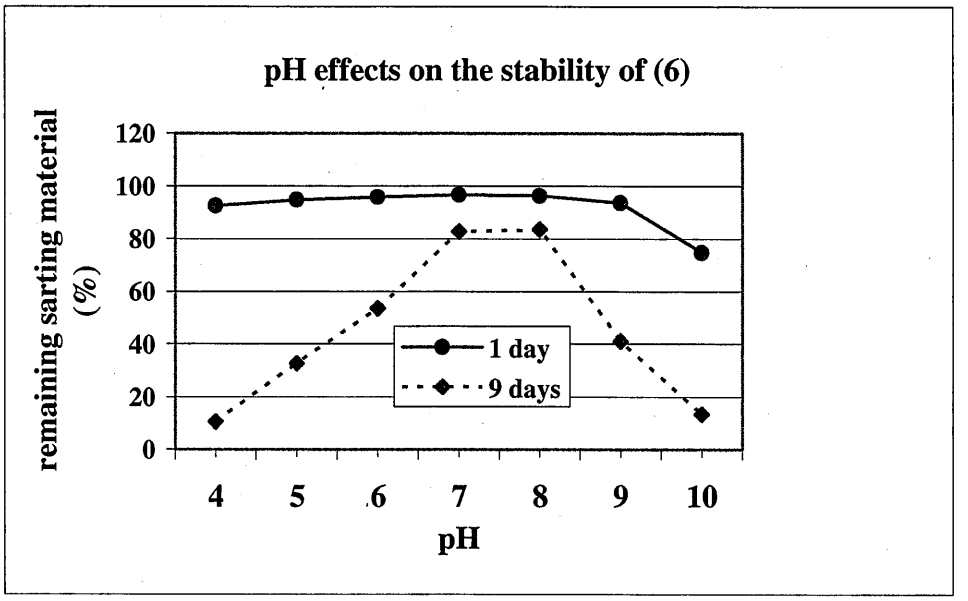
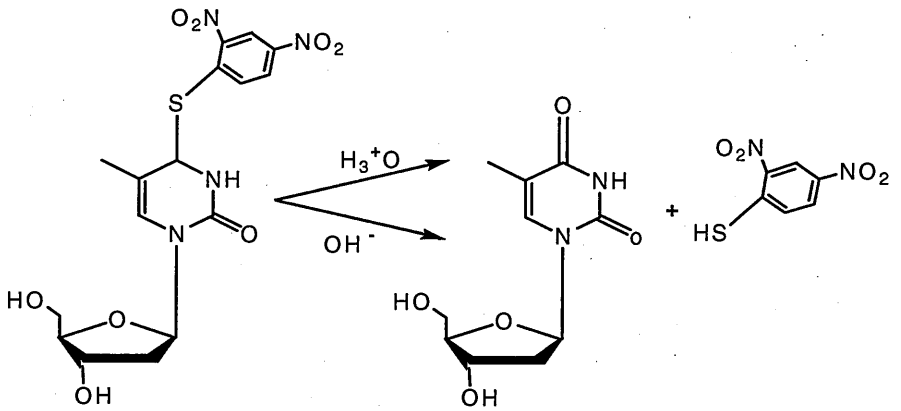


Figure 2.9 Buffers of pH 4 to10 were added to compound (6) and the effect was monitored on HPLC at different time intervals. Effect after a day and 9 days was graphically represented.

As the compound was hydrolysed at pH below 6 and at pH above 8, thus it is clear that the compound T^{SDNP} (**6**) undergoes both acid and base hydrolysis as shown in Scheme 7.

2.4.1 Characterisation of new peaks

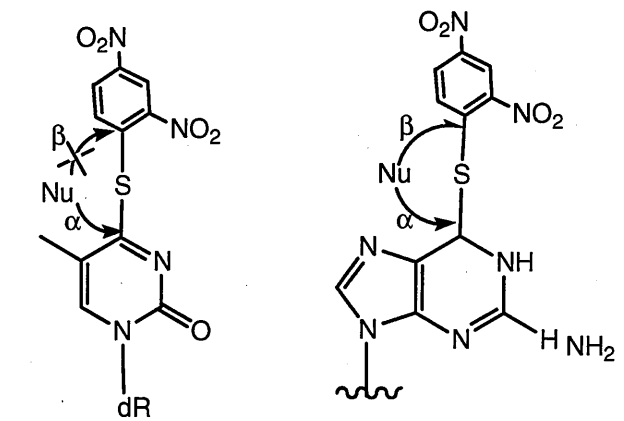
The new peaks were characterised to be that of **1** and 2,4-dinitrothiophenol (**7**) by comparing with the retention time and UV of the standards. The acid and base hydrolysis of (**6**) is illustrated in Scheme 7.



(6) (1) (7)

Scheme 7: hydrolysis of S^4 -2,4-dinitrophenythiothymidine
T^{SDNP} undergoes both acid and base hydrolysis giving the same products

Interestingly 4-thiothymidine (**5**) was not observed among the products which would suggest that **6** undergoes only nucleophilic attack at C-4 (α-attacking route) and not C-1 of the aromatic ring (β-attacking route) as reported by Xu [94] for dinitrothiopurines (see Scheme 8).



α attacking route α,β attacking route

Scheme 8: (6) undergoes α-attacking route only whereas dinitrophenyl thiopurine undergoes both α and β

The production of unwanted 6-thiopurine analogues i.e. dinitrophenyl group formation via the β-attacking route, does not make thiopurines good candidates for

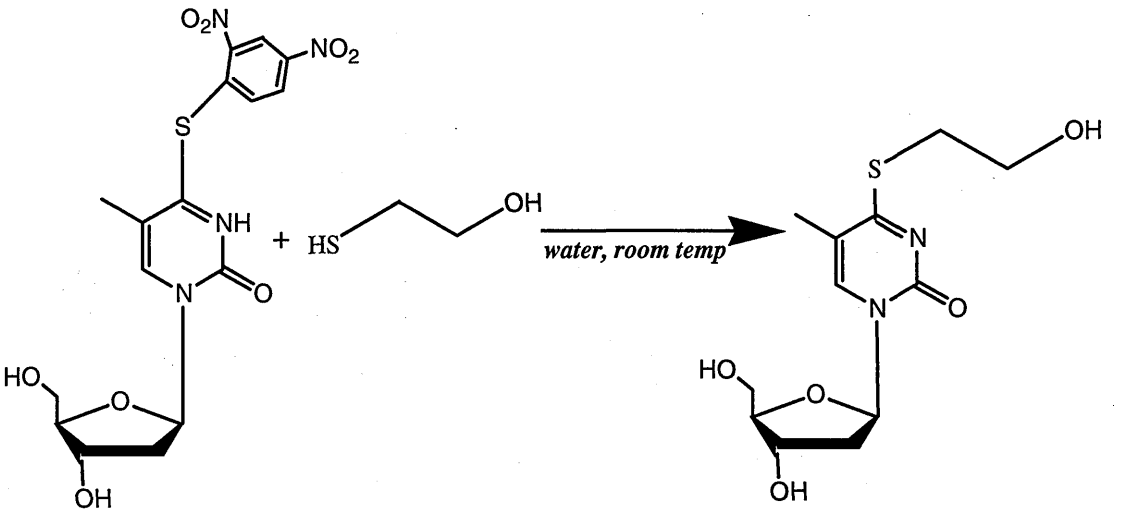
cross-linking. In contrast, the property of α-attacking only for T^{SDNP} makes it a valuable potential candidate for cross-linking.

6 was stable in aqueous solution for seven days at neutral pH. This property is compatible with its use in biological systems where the pH is around neutral. It can be stored for much longer time when dry.

To the best of our knowledge the synthesis, characterisation and the stability of **6** has not been previously reported and we explored this compound further. The good leaving ability of dinitrophenylthio group can be tested by using the very simple reagent of mercaptoethanol. It contains two nucleophilic groups a thiol and a hydroxyl. Mercaptoethanol is generally used for reducing protein disulfide bonds. It is used to cleave intermolecular disulfide bonds to allow the subunits of a protein to be separated independently. It is also used to cleave intramolecular disulphide bonds to allow denaturation of proteins.

2.5 Reaction of T^{SDNP} with mercaptoethanol

T^{SDNP} reacted very rapidly with mercaptoethanol at neutral or basic pH to form a new compound tentatively assigned as 2-hydroxyethylthiothymidine as shown in Scheme 9.



(6) **(8)**

Scheme 9: conversion of T^{SDNP} to 2-hydroxyethylthiothymidine

T^{SDNP} is completely converted to 2-hydroxyethylthiothymidine at room temperature.

The reaction was monitored by HPLC (shown in Figure 2.10). The peak of **6** was completely converted to two new peaks with UV of 460nm (out of the spectral range of Figure 2.10 (b)) for peak HS-DNP and 308nm for 2-hydroxyethylthiothymidine **8**.

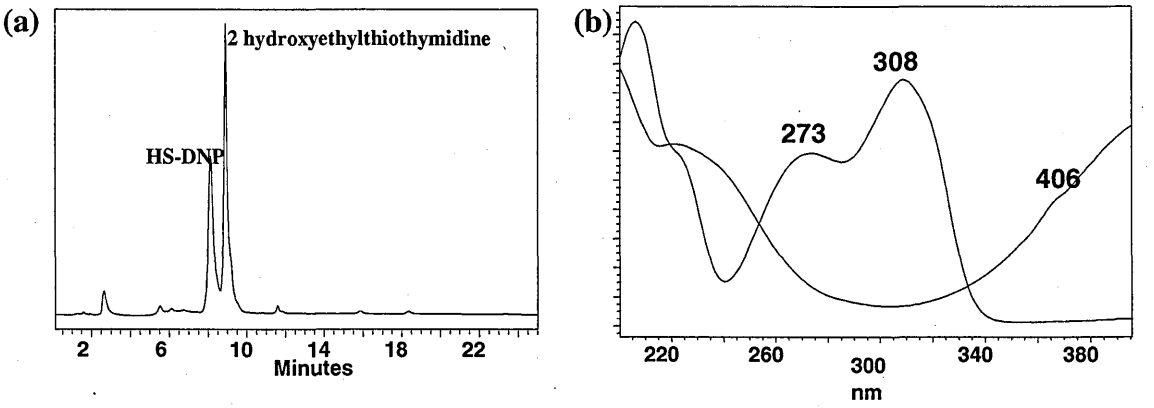
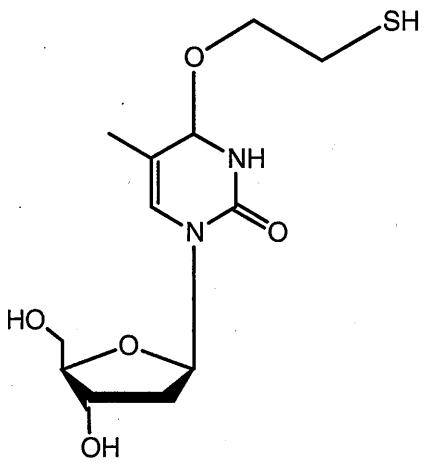


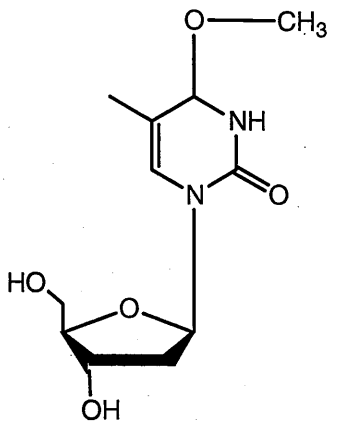
Figure 2.10 (a) immediate formation of 2-hydroxyethylthiothymidine from T^{SDNP} (b) UV of the new peaks. Atlantis^{TM} C_{18} 5μ (2.1 X 150mm) was used to monitor this reaction

UV λ_{max} of 308nm denotes the compound is S-bonded and is likely to be 2-hydroxyethylthiothymidine **8**. If the compound formed was O^4-2-

mercaptoethylthymidine **(9)** (see Scheme 9a) its UV spectrum should be similar to that of O^4-methyl thymidine **(10)**, whose λ_{max} is 281nm [107].



(9)



(10)

Scheme 9a: both the above compounds have similar structure, so should have similar kind of UV

The peak was isolated by HPLC and was subjected to mass spectroscopic analysis. For further confirmation of its structure a standard compound of **8** was synthesised. An alternative method was employed for the synthesis of the standard **8**. This involves the synthesis of 4-triazolothymidine which would then be treated with mercaptoethanol in the presence of triethylamine to form the desired product.

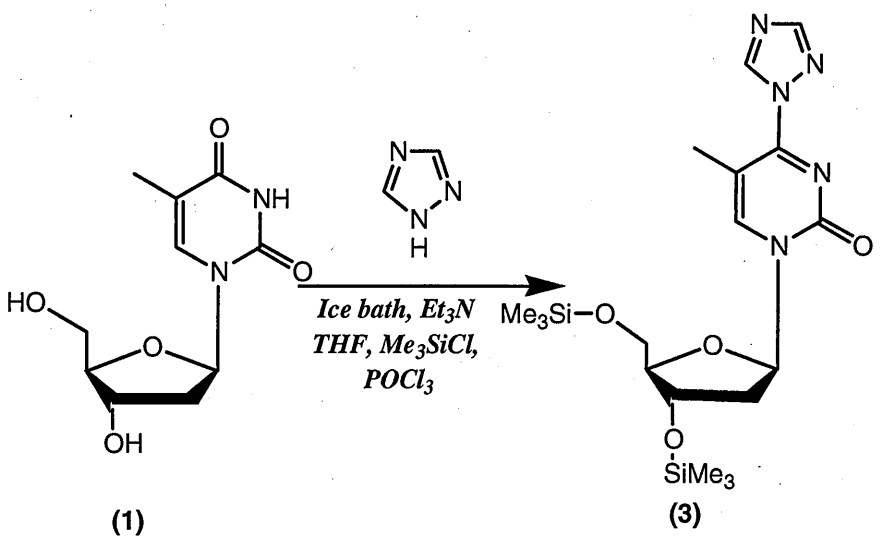
Synthesis of compound 3 by one pot reaction

2.6 Synthesis of 4-triazolothymidine (5-methyl-4-(1,2,4-triazolyl)-1-(β-2-deoxyribofuranosyl) pyrimidin-2(1H)-one, T^{TRI})

The procedure in reference [99] was followed for the synthesis of this compound which involves the following two steps

Step -1:

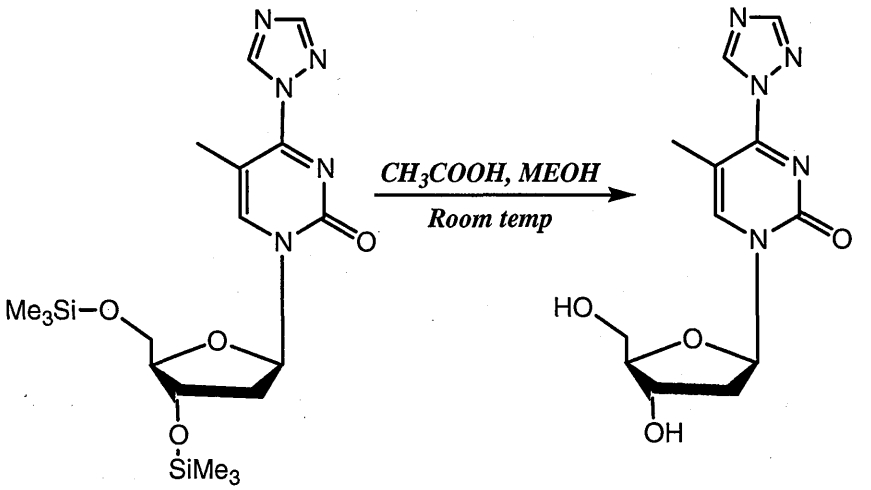
Jones's temporary trimethylsilyl protection procedure was followed to react thymidine in THF with chlorotrimethylsilane in the presence of triethylamine which afforded 3' and 5' protected thymidine[108]. This was followed immediately by triazolation by adding phosphorous oxychloride, 1,2,4-1*H*-triazole and triethylamine as described before.



Scheme 10: Protection and Triazolation were done in the same pot

Step 2:

The protecting trimethyl silyl groups were removed by treatment with acetic acid in methanol solution.



Scheme 11: deprotection was carried out by adding 1:4 acetic acid and methanol stirring at room temperature overnight

The purity of **11** was checked on HPLC and a single peak was obtained (Figure 2.11) and used for synthesising the following standards

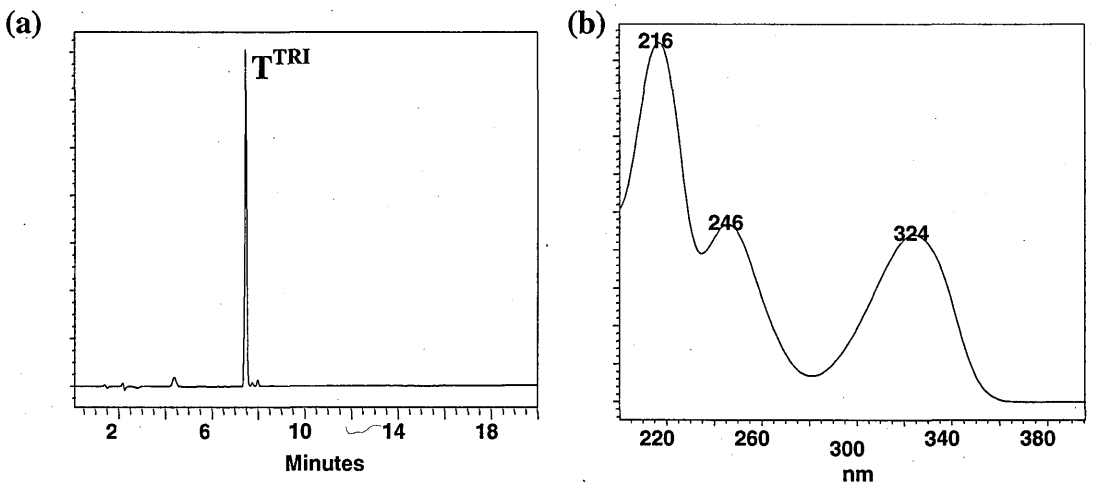
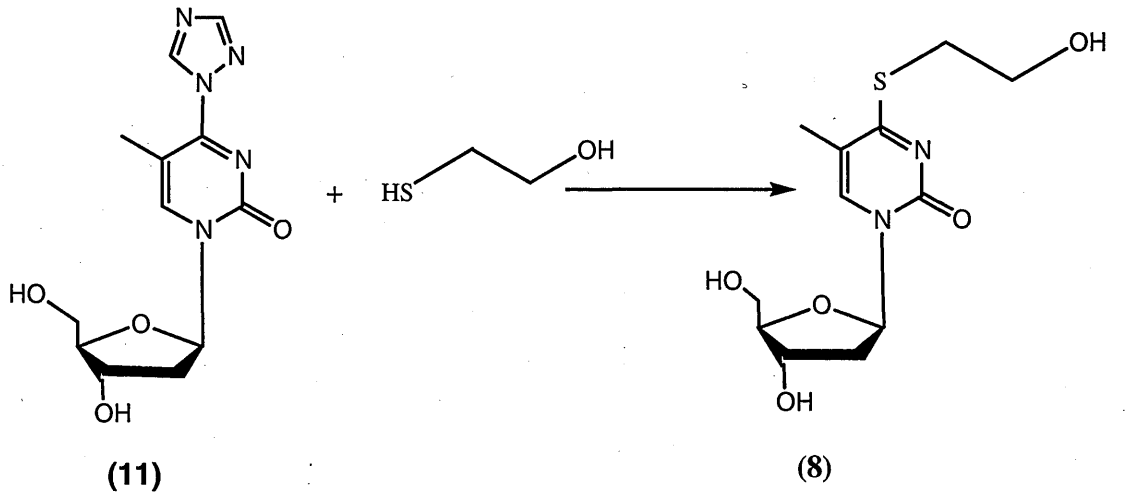


Figure 2.11 (a) single peak of T^{TRI} (b) UV of T^{TRI}

2.7 Synthesis of standard 2-hydroxylethylthiothymidine

11 in methanol was reacted with mercaptoethanol in the presence of triethylamine at room temperature to give **8.**



(11) **(8)**

Scheme 12 Standard 2-hydroxyethylthiothymidine was synthesised by reacting with mercaptoethanol at room temperature in presence of triethylamine

Pure **8** was obtained following silica gel column chromatography. The purity of the compound was determined by HPLC as shown in figure 2.12.

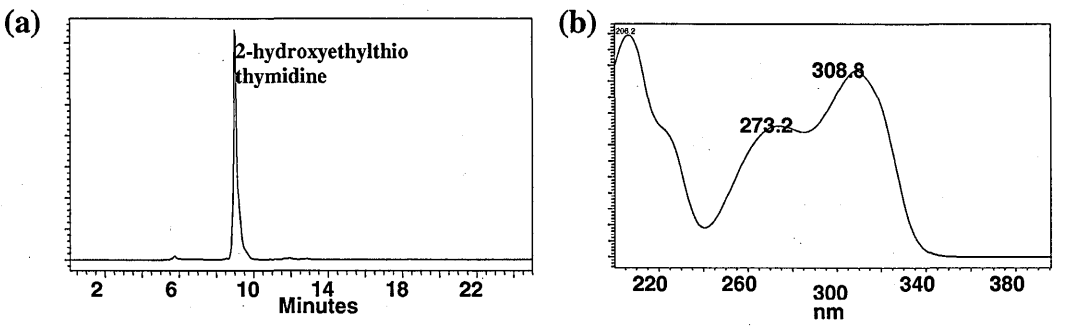


Figure 2.12 (a) highly pure 2-hydroxyethylthiothymidine was obtained (b) UV

NMR data was obtained which confirms the structure of the compound as shown below. The compound was subjected to mass analysis which was similar to that of the previously isolated peak of **8.**

^{1}H NMR Data (in DMSO-d_6): 1.95 (3H, s, 5-methyl), 2.01-2.28 (2H, m, 2' and 2"-H), 3.21 (2H, t, α-methylene), 3.60 (4H, m, 5'-H, and β-methylene), 3.83 (1H, dd, 4'-H), 4.21 (1H, m, 3'-H), 5.01 (1H, t, 5'-OH), 5.24 (1H, d, 3'-OH), 6.06 (1H, t, 1'-H), 7.9817 (1H, s, 6-H).

^{13}C NMR (in DMSO-d_6) 13.67 (5-CH_3), 59.35 (C-5'), 60.81 (β-C of OH), 69.77 (C-3'), 85.17 (C-1'), 87.73 (C-4'), 110.44 (C-5), 138.60 (C-2), 152.43 (C-6), 176.2 (C-2)

UV λ_{max}: 308.8;

MS *m/z*: 302.88 (M) 186.9 [(M+ H) - sugar) and the mass fragments were obtained under electro spray conditions. The observed fragments were M^+ 302, M^+-H 301 and M^+H 303.

For further evidence that the synthesised standard and the product formed by the reaction of **6** with mercaptoethanol were same, the standard **8** was co-injected along with the reaction mixture of **6** and mercaptoethanol on HPLC. The observations were as follows:

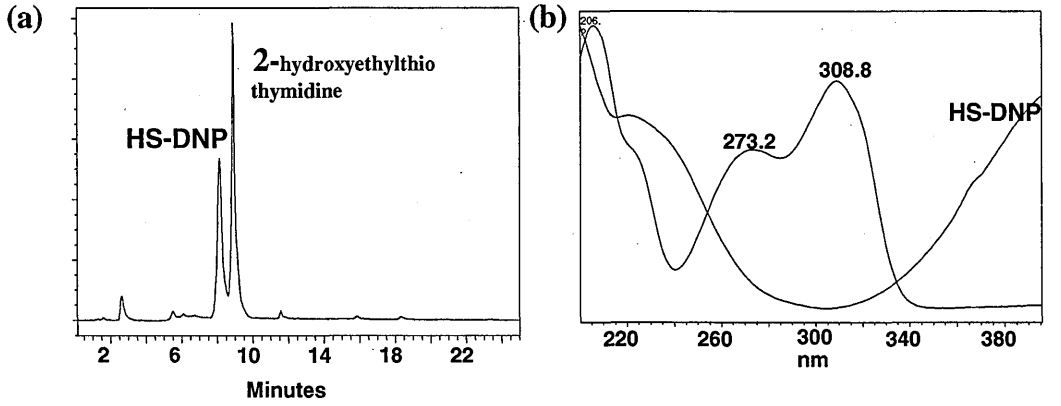


Figure 2.13 (a) HPLC trace of reaction of T^{SDNP} with mercaptoethanol (b) UV λ_{max} of both peaks

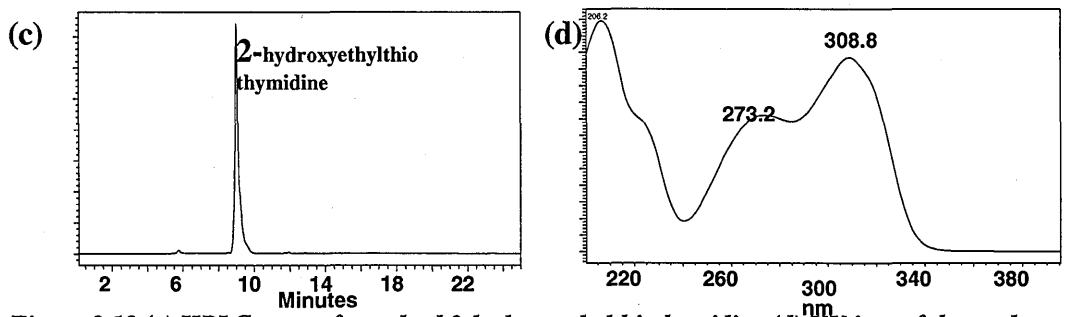


Figure 2.13 (c) HPLC trace of standard 2-hydroxyethylthiothymidine (d) UV λ_{max} of the peak

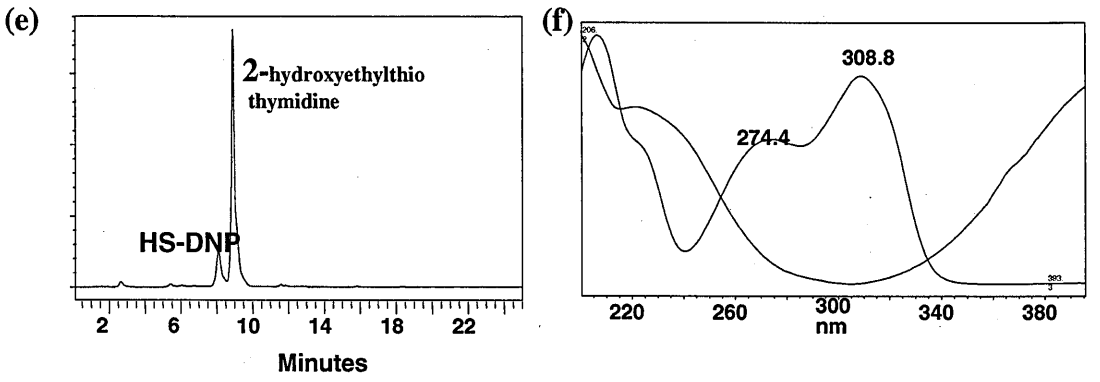
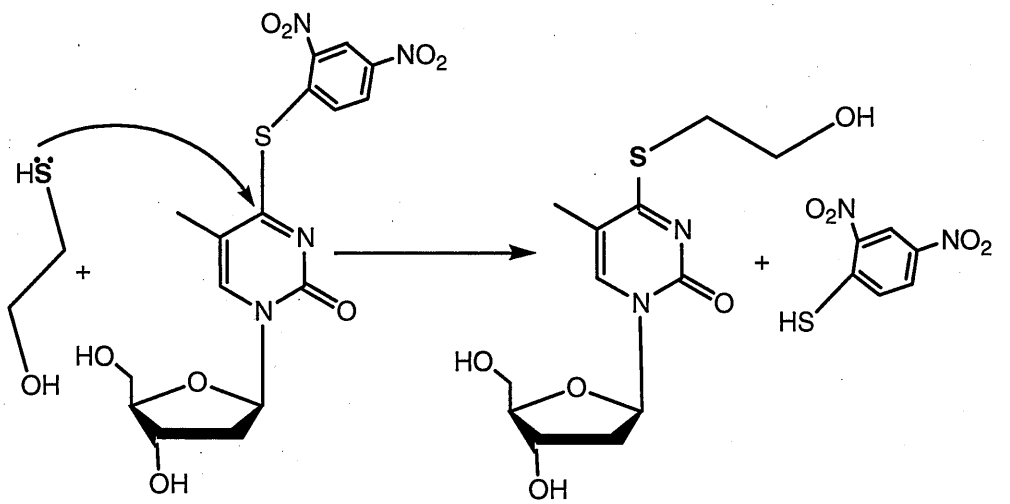


Figure 2.13(e): Co –injection of standard 2-hydroxyethylthiothymidine with reaction mixture of T^{SDNP} and mercaptoethanol. Peak of 2-hydroxyethylthiothymidine was increased with the coinjection. So both synthesised standard and the product formed from mercaptoethanol and T^{SDNP} are same.

Possible Reaction Mechanism



Scheme 11 thiol attack on T^{sdnp}

The thiol agent is a strong nucleophile and can attack the C-4 of **6** to form **8**. The sulphur of the formed product was thought to be from mercaptoethanol rather than from 4-thiothymidine (**5**). This could be confirmed by the fact that **5** was not

observed in this reaction and as discussed earlier **6** undergoes only α-nucleophilic attack.

2.7.1 Characteristics of 2-hydroxyethylthiothymidine

The compound was found to be stable in water for an hour as shown in Figure 2.14 (a). However 75% of the compound was hydrolysed after five days as shown in Figure 2.14 (**c**). The new peak has UV λ_{max} at 267nm, which is characteristic of **1**. Co-injection of standard **1** would confirm the hydrolysed product (see figure 2.14) (**g**).

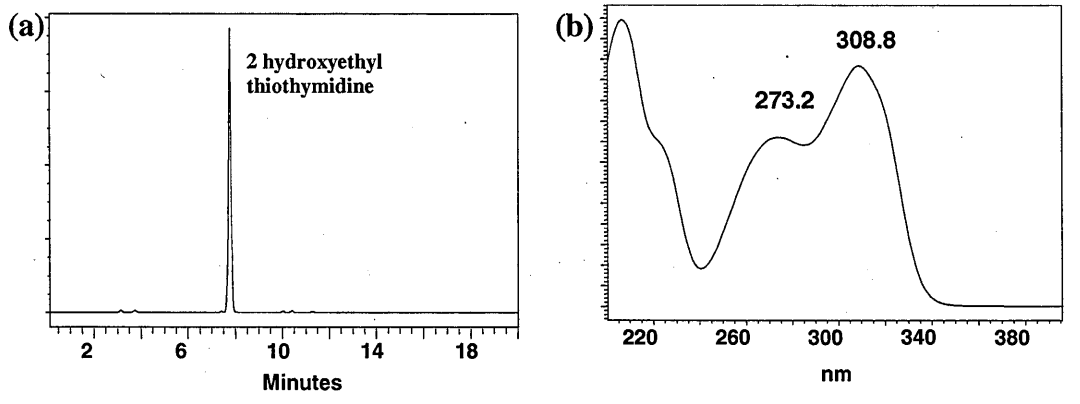


Figure 2.14 (a) HPLC trace of compound 8 in water after 1 hour (b) UV λ_{max} of compound 8

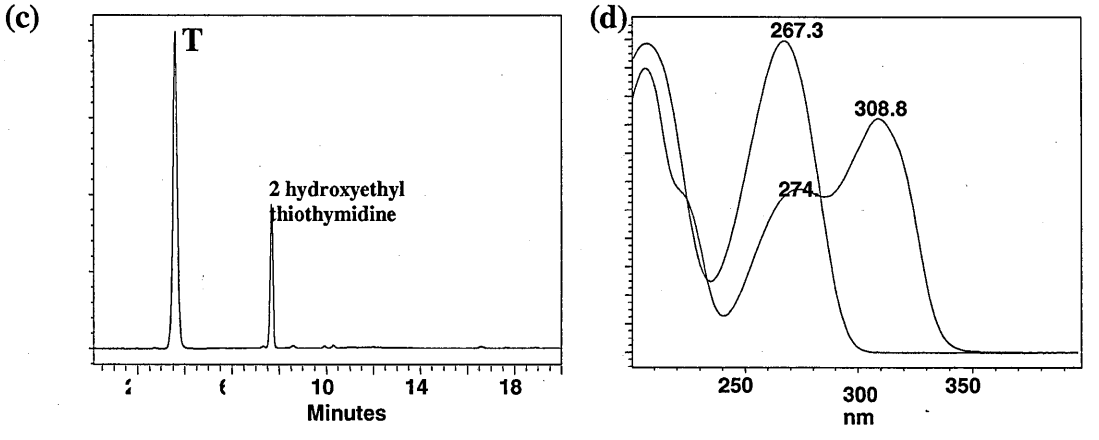
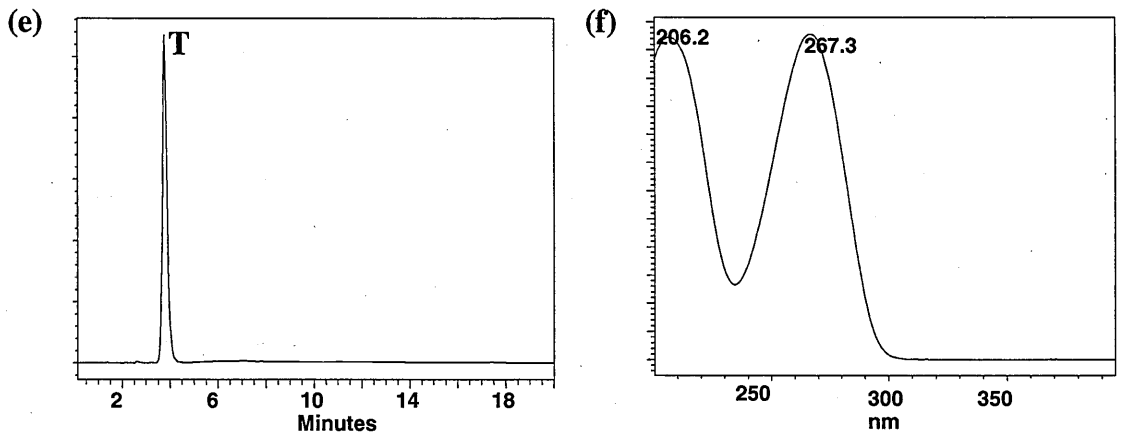


Figure 2.14 (c) HPLC trace of compound 8 in water after 5 days (d) UV λ_{max} of peaks



2.14 (e) HPLC trace of standard thymidine (f) UV λ_{max} of thymidine

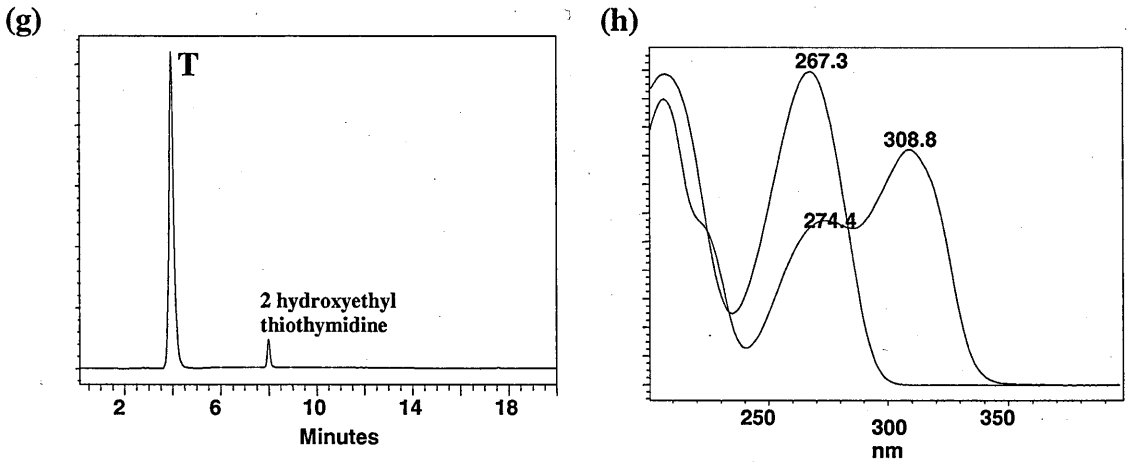
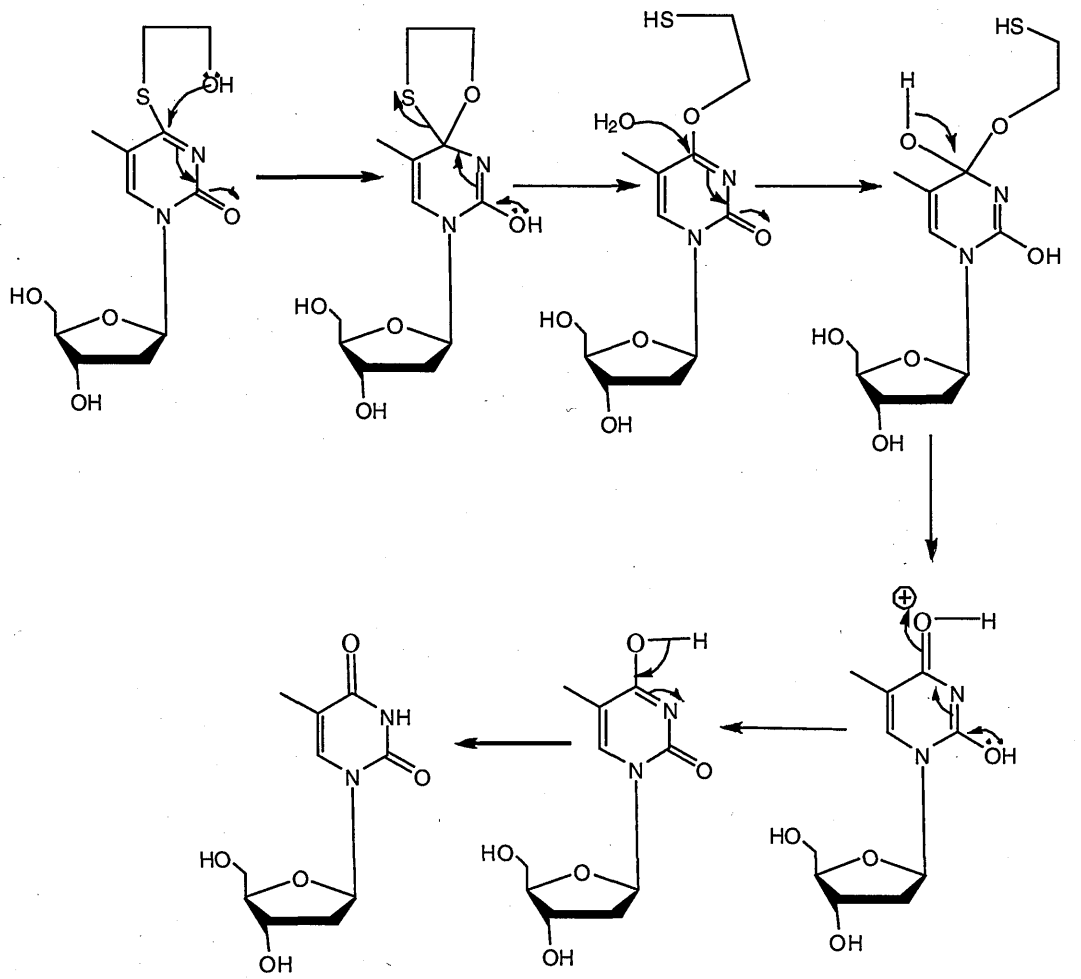


Fig 2.14 (g) HPLC trace of, standard thymidine was injected into 5 day 2-hydroxyethylthiothymidine mix. Thymidine peak is increased compare to 2-hydroxyethylthiothymidine. This confirms the extra peak formed from the hydrolysis of compound 8 is thymidine.

So the above experiment confirms that the new peak formed was that of compound **1**. One possible explanation for the formation of compound **1** is the effect of neighbouring nucleophile as illustrated in Scheme 12.

Possible reaction mechanism



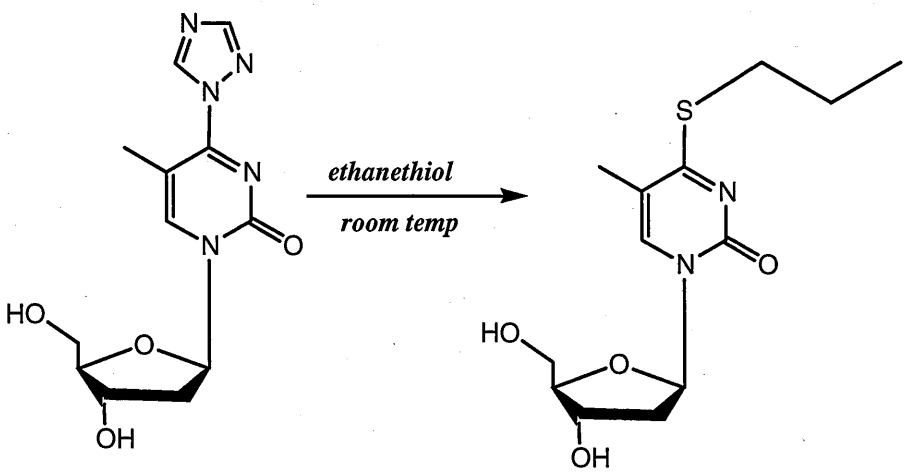
Scheme 12 : The neighbouring OH group on 2-hydroxyethylthiothymidine attacks the C4 of (5) that would eventually lead to the formation of (1)

Due to the neighbouring effect, the hydroxyl group in the side chain would attack the C4 of **5** which might lead to the cyclisation and formation of a temporary five membered ring. Due to the presence of excess of water in the surrounding environment the oxygen atom of mercaptoethanol is replaced by oxygen of water which leads to the formation of **1**. This kind of neighbouring effect would be seen only if there were other nucleophiles in the molecule. Synthesising a structurally similar compound without a neighbouring nucleophile in its structure would provide evidence for this mechanism. For this purpose ethanethiol was chosen as the reagent. Ethanethiol is similar to mercaptoethanol except for a hydroxyl group. So the final

product formed would have the same structure as that of **8** except for the hydroxyl group.

2.8 Synthesis of 4-ethylthiothymidine

4-Ethylthiothymidine (**12**) was synthesised from **9**, by the similar methodology used for the synthesis of **8**.



(9) **(12)**

Scheme 13: Compound (**9**) *in methanol was subjected to react with ethanethiol at room temperature in the presence of triethylamine to form* (**12**)

The reaction was monitored by HPLC as shown in Figure 2.15

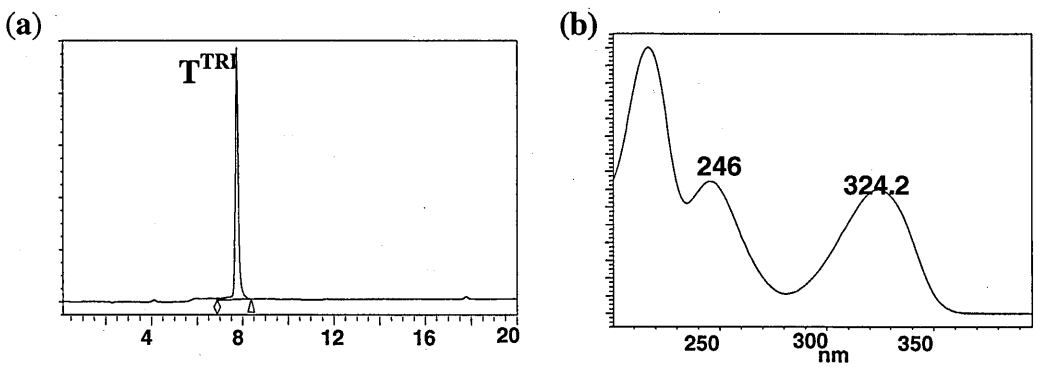


Figure 2.15 (a) HPLC trace of standard triazolothymidine (b) UV λ_{max} of T^{TRI}

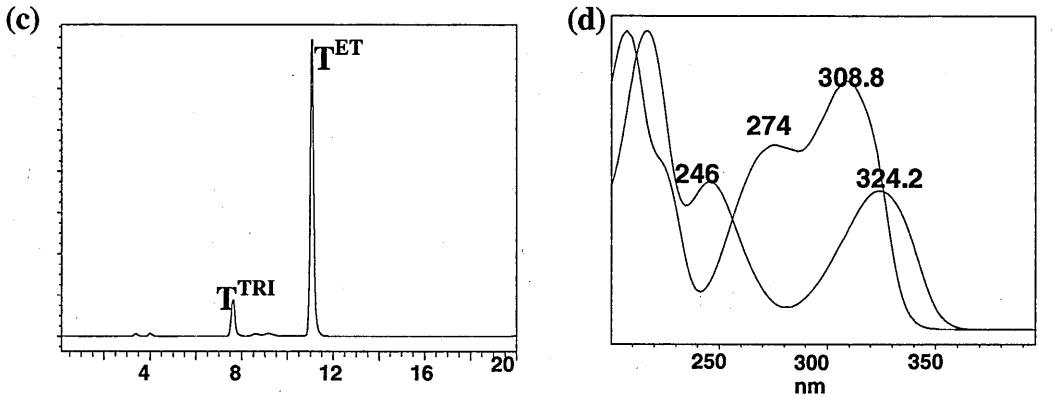


Figure 2.15 (c) HPLC trace of T^{TRI} converting to ethylthiothymidine with the addition of ethanethiol. (d) UV λ_{max} of both peaks

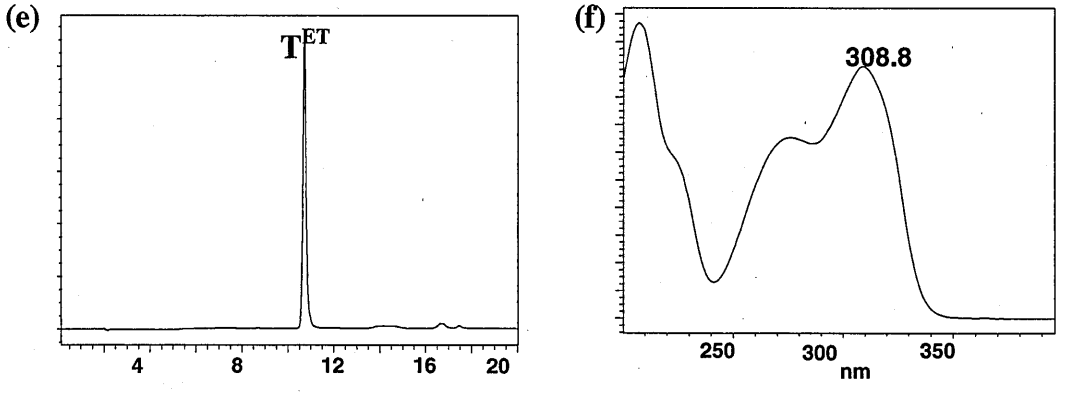


Figure 2.15 (e) 4-Ethylthiothymidine was isolated and dried. The reaction was left for completion (figure not shown). It was dissolved in water and injected after five days fig 2.15 (f) UV λ_{max} of T^{ET}

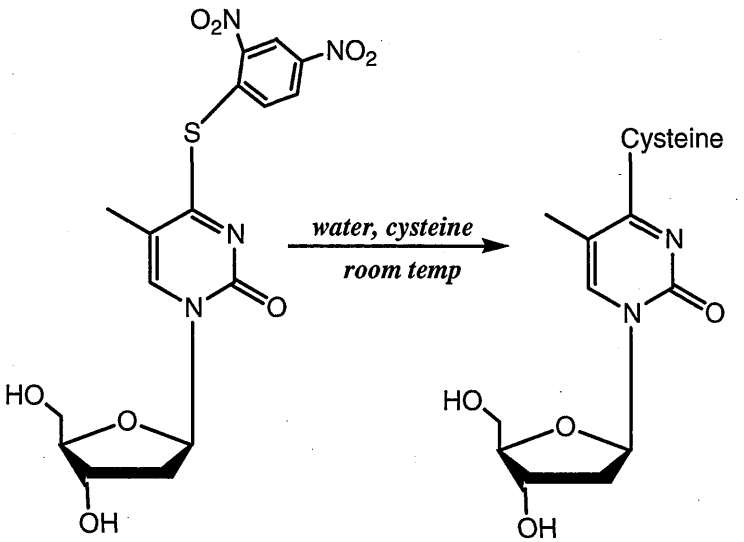
Compound **12** is stable compared to **8**. This is due to the absence of neighbouring nucleophilic hydroxyl group. This not only proves that the presence of hydroxyl group in **8** makes it more susceptible to water but that the compound **8** is also S-bonded.

So with these studies it could be concluded that, of the different nucleophiles within the same molecule the thiol functional group reacts faster with **6**. For site specific cross-linking, the ideal reagent would be of biological origin and containing a unique nucleophile that can displace dinitrophenyl group of **6**. Of the natural amino acids

cysteine is the only amino acid with thiol group in its molecule along with an amino group. This unique property of cysteine could be exploited for cross-linking.

2.9 Amino acid cross-linking: Reaction of T^{SDNP} with cysteine

6 reacts with cysteine rapidly in aqueous solution to give a new product which was believed to be 4-cysteinylthymidine (**13**). However, at this stage the exact structure of **13** was not clear so that it was not explicitly drawn out in Scheme 16

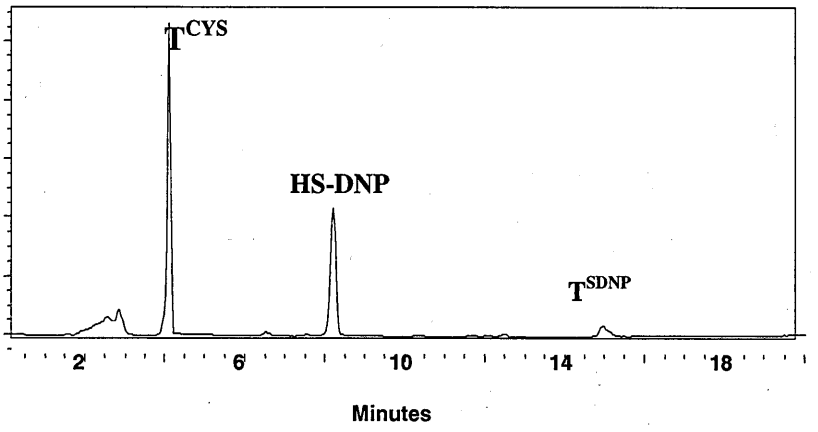


(6) (13)

Scheme 16: Compound **6** *reacts with cysteine and forms 4-cysteinylthymidine at room temperature*

The reaction was rapid at room temperature when monitored on HPLC (see Figure 2.16).

(a)



(b)

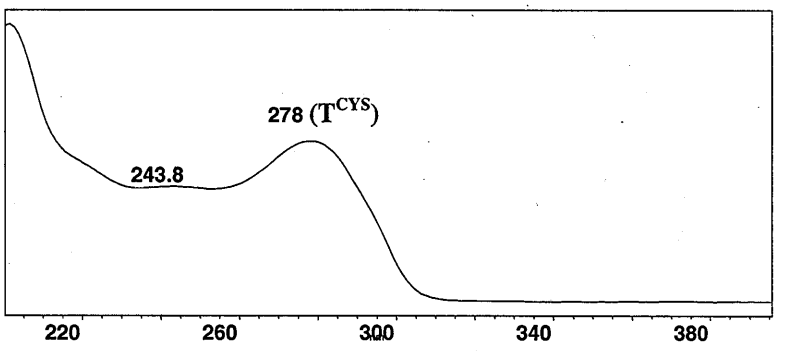


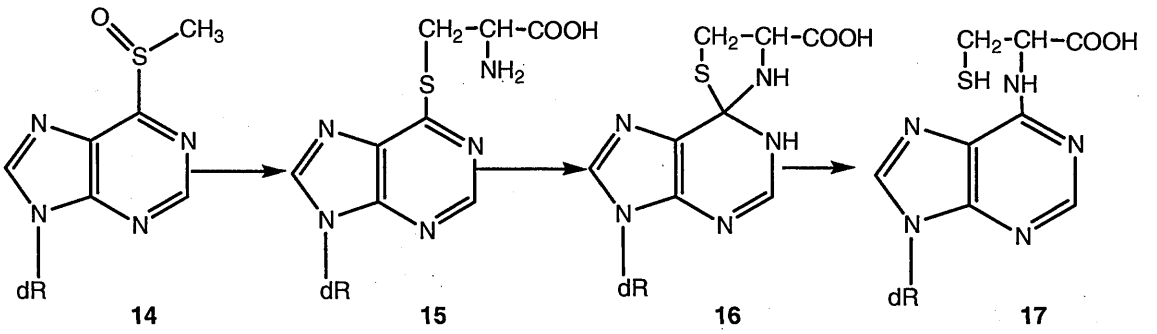
Figure 2.16: Cysteine was added to compound 6 and injected immediately at room temperature (a). UV of T^{CYS} (b). UV of other peaks not shown.

Interestingly the new peak formed has a λ_{max} at 278nm as shown in Figure 2.16 *(b)*, similar to the previous observation the S-bonded **8** has UV λ_{max} at 308nm. Also **5** and **6** which were S-bonded have UV λ_{max} of more than 300nm as shown in table 1. So, is **13** S-bonded or N-bonded?

4S bonded Compounds	UV λ_{max} (nm)
4-Thiothymidine **5**	335
T^{SDNP} **6**	315
2-Hydroxyethylthiothymidine **8**	308.8
Ethylthiothymidine **12**	308.8

Table 1 representing UV λ_{max} of various 4S-bonded thymidine derivatives

In an earlier report by Xu [88], a mechanism was proposed for the formation of N-bonded, 6-cysteinylpurine-2'-deoxynucleoside from the reaction between 6-methylsulphoxypurine-2'-deoxynucleoside and cysteine . The proposed mechanism for the formation of the N-bonded compound is shown in scheme *17*.

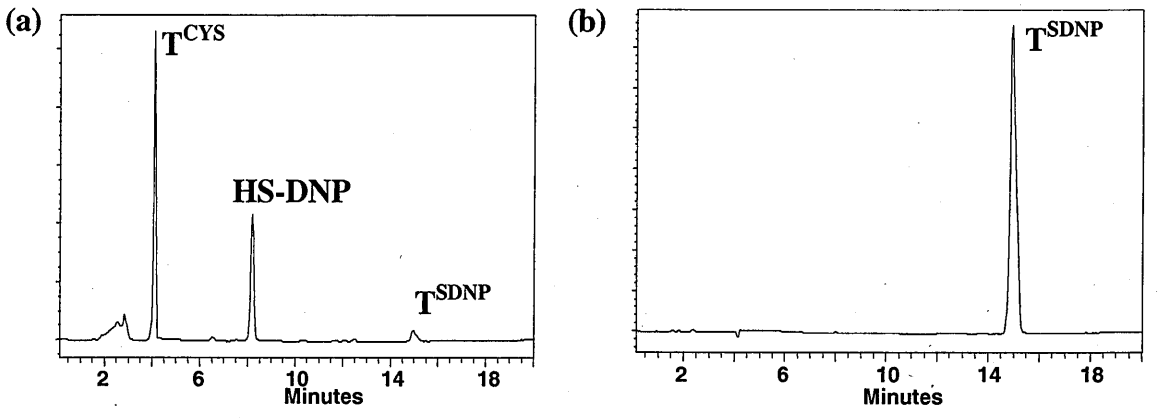


***Scheme 17 mechanism for transformation of* 14 *to* 17**
(adapted from Xu, Tetrahedron, 54: 187-196)

According to the report the 6-methylsulphoxypurine-2'-deoxynucleoside (**14**) reacts with cysteine to form a putative S-bonded 6-cysteinylpurine-2'-deoxynucleoside intermediate (**15**) which was rapidly converted to another intermediate (**16**). The intermediate (**16**) slowly converts to N-bonded 6-cysteinylpurine-2'-deoxynucleoside (**17**). Purification of the intermediate **15** by HPLC was reported and as was the conversion of purified intermediate into the cyclic intermediate **16** and the product **17**.

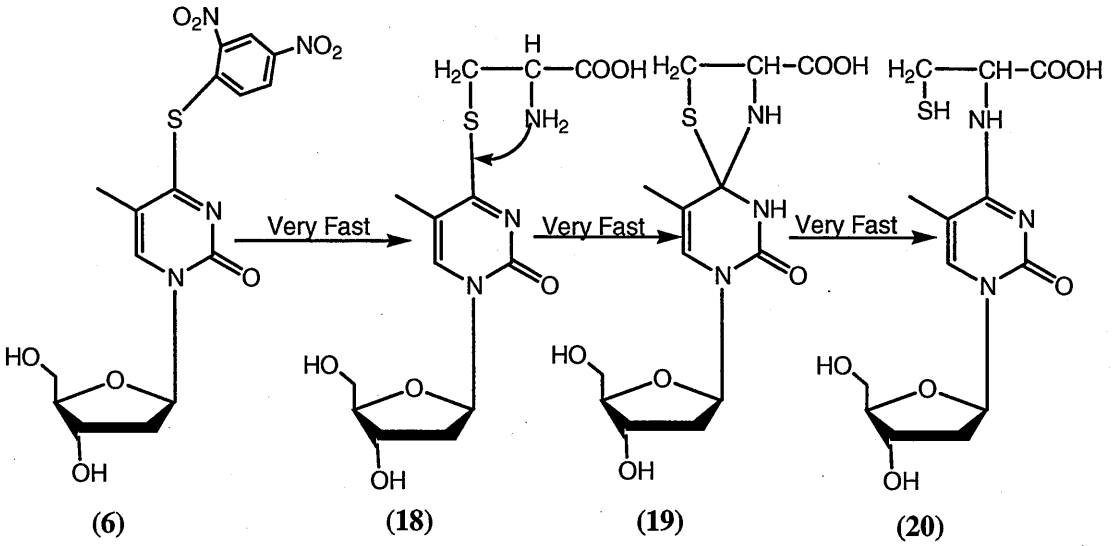
To support the proposed mechanism it was reported that there was no reaction observed between **14** and glycine (simplest amino acid with amino group) under the same reaction conditions as with cysteine.

However, in the reaction of **6** with cysteine no intermediates were observed when the reaction was monitored on HPLC at room temperature. For comparison the reaction of glycine was performed and no products were observed as shown in Figure 2.17 (b). This finding is similar to the case of the modified purine analogue i.e., **14**, reported by Xu [88]. Glycine is the simplest amino acid with an amino and carboxyl group. Cysteine and glycine were added separately to **6** in the presence of phosphate buffer (pH 6.5) at room temperature and the reaction was monitored by HPLC.



2.17 No reaction with glycine was observed (b) as opposed to cysteine (a) where the reaction was rapid at room temperature

6 was converted immediately to a new peak of **13** (see Figure 2.17 *(a)*) while new peaks were not observed even after leaving for overnight with glycine as seen in Figure 2.17 *(b)*. This leads to the conclusion that the thiol of cysteine would attack the C4 of **6** first as shown in Scheme 18 and the same pattern as reported by Xu [88] might occur.



Scheme18: Probable mechanism for the formation of N-bonded 4-cysteinylthymidine

To examine the above hypothesised mechanism another set of experiments were carried out where slightly less than equimolar concentration of cysteine was added to **6** as mentioned earlier. The reaction was monitored by HPLC and the temperature of the reaction was maintained at 5°C through out the reaction. This setting up of temperature to 5°C was made possible by using Waters™ 2690 HPLC instrument. The observations were as follows

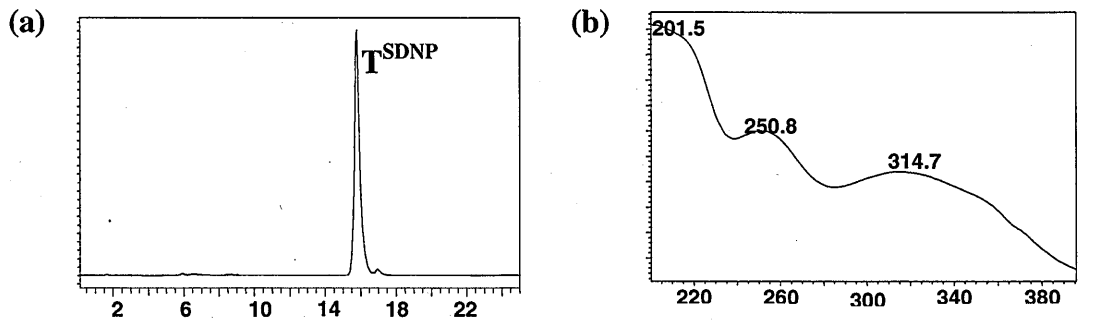


***Figure 2.18:* 6 *was already at* 5°C for 20 min (a) and the UV (b)**

To the above pre-cooled **6**, cysteine was added and injected immediately. The following pattern was observed.

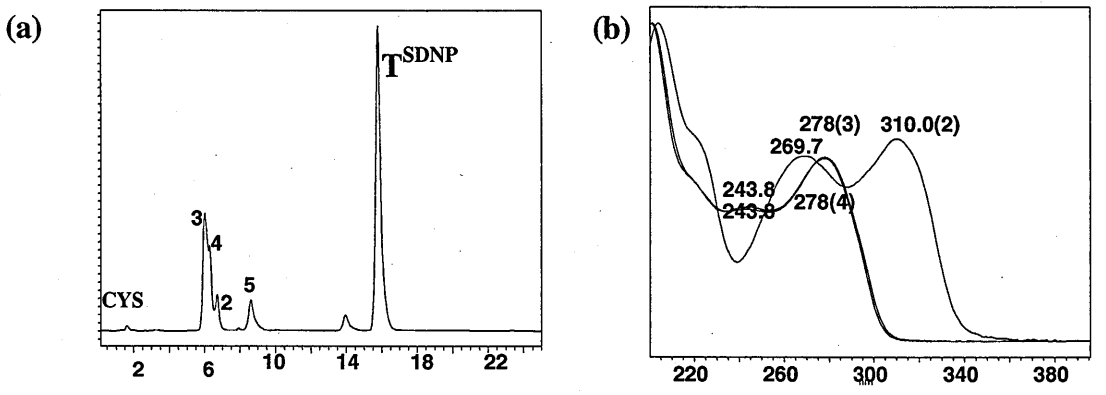


Figure 2.19 (a) **6** *was immediately converted to new peaks on addition of cysteine. (b) UV of new peaks- designated as 3,4,5. (CYS for cysteine)*

Peak 2 in Figure 2.19 (a) has λ_{max} at 310nm. This would appear to be the peak of S^4-cysteinylthymidine **18** based on the data in Table 1 showing that all S^4-bonded thymidine derivatives show a maximum UV absorption above 300 nm. This was also reported in various literature references [76, 109]. S^4-bonded 4-thio-5-bromodeoxyuridine has λ_{max} of 335nm and 4-thiothymidine was shown to have λ_{max} of 330nm. Other known S^4-bonded derivatives of thymidine such as 4-glutathionylthiothymidine (see below) have UV λ_{max} around 310 nm as shown in Figure 2.20. Peaks 2, 3 and 4 were not isolated.

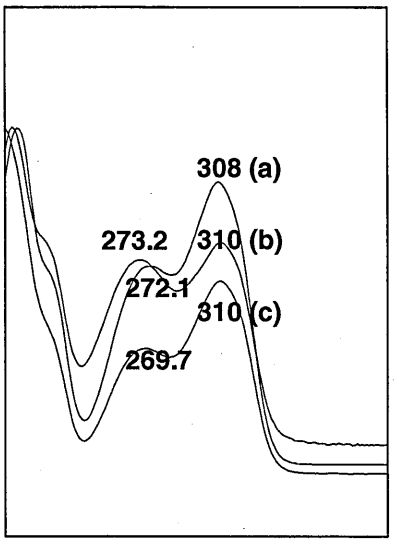
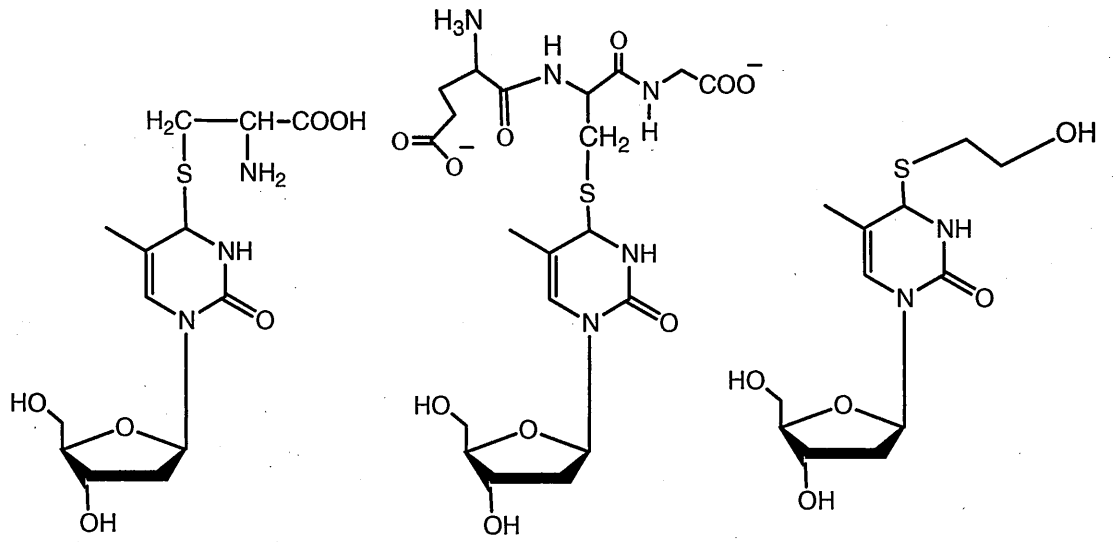


Figure 2.20: UV of S^4-thymidine derivatives. Peak (a) 2-hydroxyethylthiothymidine, peak (b) 4-cysteinylthymidine and (c) 4- Glutathionylthymidine. All the peaks have similar UV patterns

The S^4-bonded compounds such as glutathionylthiothymidine, 2-hydroxyethylthiothymidine and S^4-cysteinylthymidine have structural similarity as shown below and have the same UV patterns as shown in figure 2.21.



S^4-cysteinylthymidine **S^4-glutathionylthymidine** **ß-hydroxyethylthiothymidine**

Figure 2.21 Chemical structure of S^4-bonded thymidine compounds

Thus peak 2 can be tentatively assigned as S^4-cysteinylthymidine **18**.

Peak 3 and 4 have the same UV maximum absorption at 278nm. This is characteristic feature of N^4-bonded thymidine derivatives. **21** has maximum UV absorption at 278 nm. All N^4-bonded thymidine derivatives have UV around 280 as shown in Figure 2.23. So it is reasonable to assume that peak 3 and 4 are N^4-bonded cysteinylthymidine derivatives.

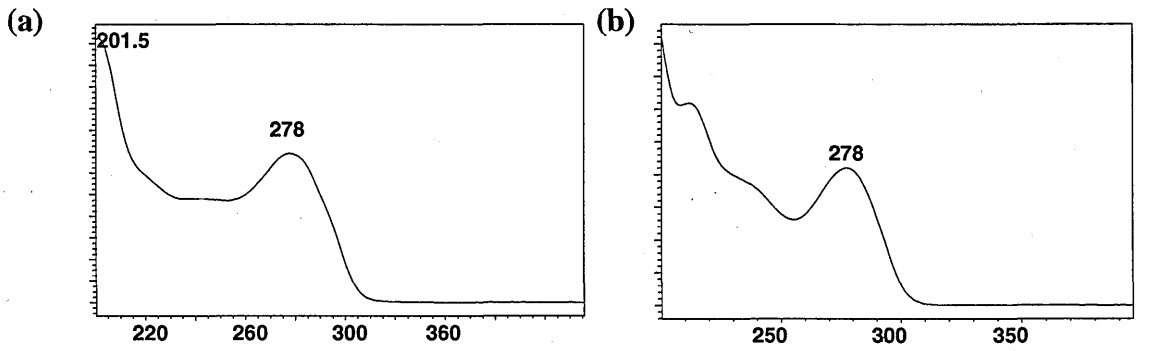
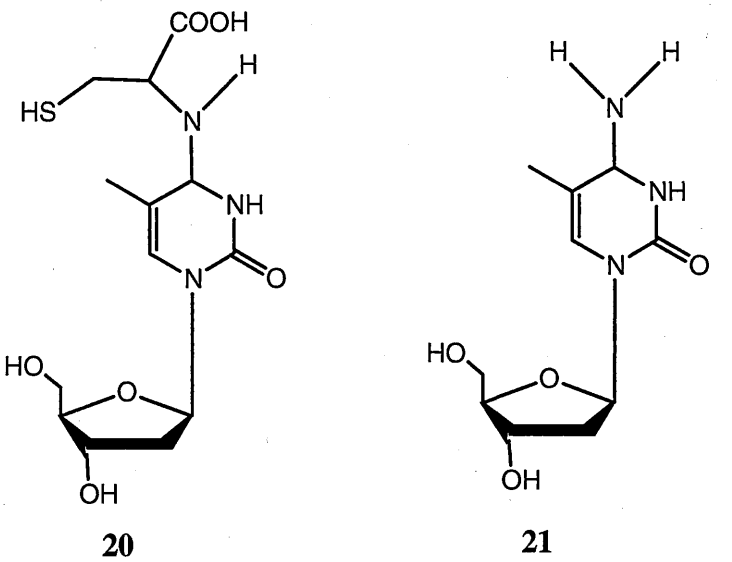


Figure 2.23 (a) UV of N^4-cysteinylthymidine (b) UV of 5-methylcytidine

5-Methyl cytidine (**21**) is structurally similar to N^4-cysteinylthymidine (**20**) as shown in Figure 2.22.



20 **21**

Figure 2.22 Structure of N^4-bonded thymidine derivatives

When the reaction was monitored after an hour peak 2 disappeared completely and peak 3 remained as the major peak as shown in Figure 2.24. At this stage the starting material (T^{SDNP}) peak also disappeared.

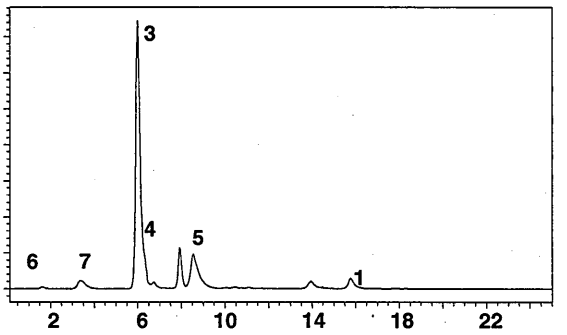


Figure 2.24: the reaction was monitored after an hour.
The reaction was maintained at 5°C

Peak 5 was assigned as compound 7 (i.e. the leaving group) which has UV λ_{max} at 406nm. Peak 6 was assigned as cysteine which was confirmed by injecting a standard of cysteine. When the reaction was left for overnight and monitored peak 3 was slowly converted to peak 4 which has similar kind of UV.

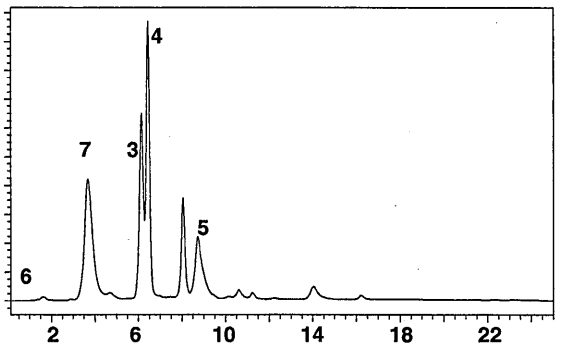
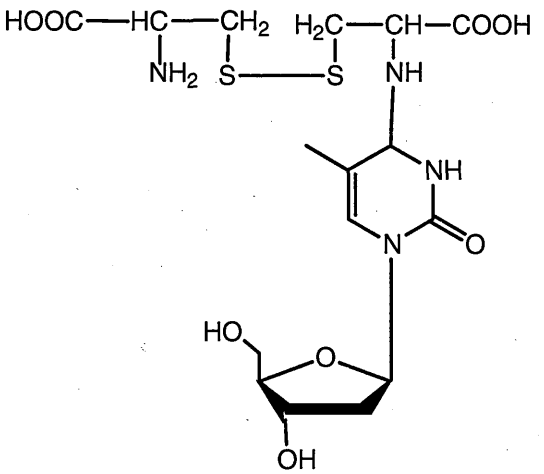


Figure 2.25: after overnight peak 3 was converted to peak 4.
The reaction temperature was still maintained at 5°C

Peak 7 has increased in size after leaving the reaction mixture for overnight with UV λ_{max} of 278nm which is again characteristic of N^4-bonded thymidine derivatives as discussed earlier. This peak was not observed on immediate injection of the reaction

mixture and developed into another major peak after leaving the reaction overnight. The retention time of this peak was at 2.5 minutes, implying, a highly polar compound, as highly polar compounds elute first on reversed phase column. This peak was observed only in the presence of free cysteine in the reaction mixture. There data were consistent with peak being $T^{cys—cys}$ (4-cystinylthymidine **22** as shown in figure *2.24*) as excess cysteine in the reaction mixture is likely to slowly form a disulphide link with N^4-cysteinylthymidine.



22

Figure 2.26 Structure of 4-cystinylthymidine ($T^{cys-cys}$)

In the absence of excess cysteine peak 7 (**22**) was not observed as shown in figure 2.27.

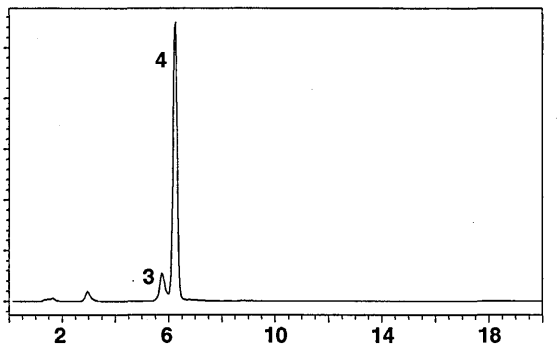


Figure 2.27: N^4-cysteinylthymidine isolated peak

Peak 4 has UV absorption maximum at 278 which is exactly similar to that of peak 3 suggesting that it was a similar type of compound. The retention time was slightly later than that of peak 3 which suggested that it was less polar. Taking into consideration the fact that free thiol groups are susceptible to oxidation and usually generate disulfides, the free thiol group of N^4-cysteinylthymidine might form disulphide bridges among themselves to form a dimer ($T^{cys\text{-}cys}T$) **23**. Further support for this proposal came from the fact that peak 3 was completely converted to peak 4 after four days as shown in the Figure 2.28.

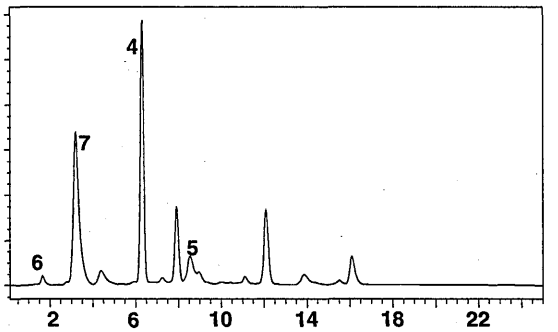


Figure 2.28: after 4days peak 3 was totally converted to peak 4

To confirm that peak was that of **23** a separate set of experiments were performed to see if the disulphide bonds could be broken to regenerate peak 3 .

2.9.1 Reducing disulfide bonds

Dithiothreitol (DTT), also known as Cleland's reagent, contains two thiol groups and is widely used in biochemical studies for reducing disulphide bonds of proteins.

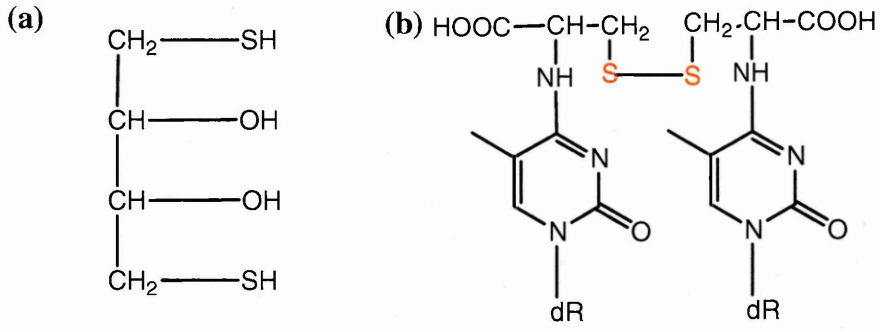
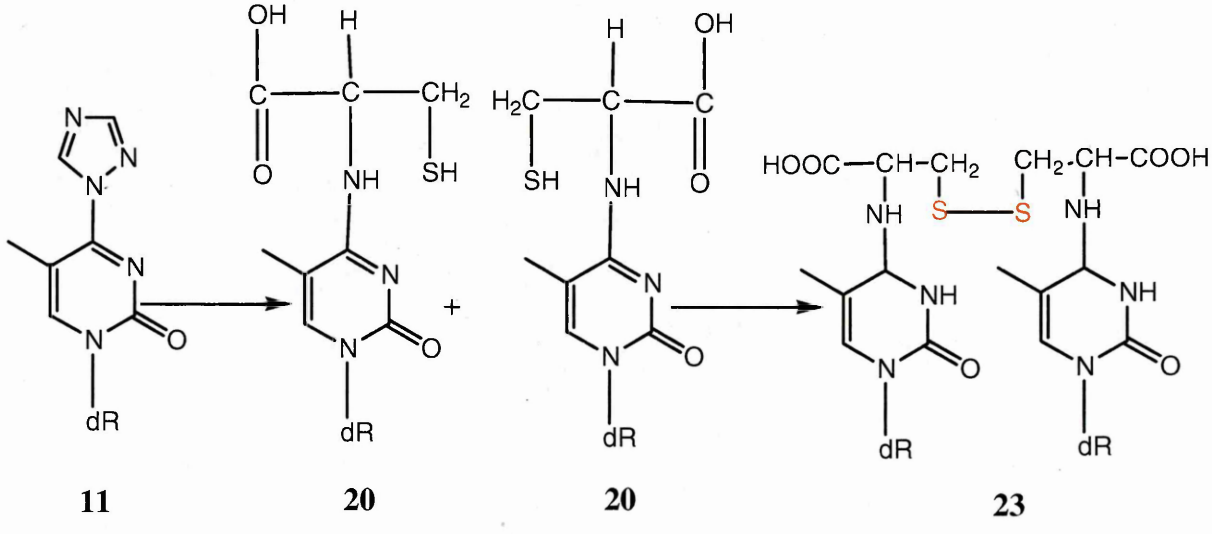


Figure 2.29 (a) 1,4-Dithiothreitol,(1,4-dimercapto-2 ,3-butanediol)

(b) N^4-cysteinylthymidine dimer ($T^{cys\text{-}cys}T$)

If the proposed structure for peak 4 is correct (as figure *2.29 (b)*) then it should be reduced to peak 3 on addition of DTT and the new peak should be eluted at earlier time without any change in the UV λ_{max}. For this purpose an authentic **23** standard was synthesised using **11**.

Compound **11** in water reacts with cysteine in the presence of triethylamine at room temperature and forms **20**. **20** subsequently dimerises, forming disulphide linkages among themselves to form compound **23.**



Scheme 19: **11** *in water reacts with cysteine in the presence of triethylamine at room temperature to form* **20.** *the formed* **20** *would form disulphide bonds to form* **23**

When the reaction was monitored by HPLC, interestingly a peak with UV λ_{max} at 310nm was first observed as shown in figure *2.30* and then disappeared immediately.

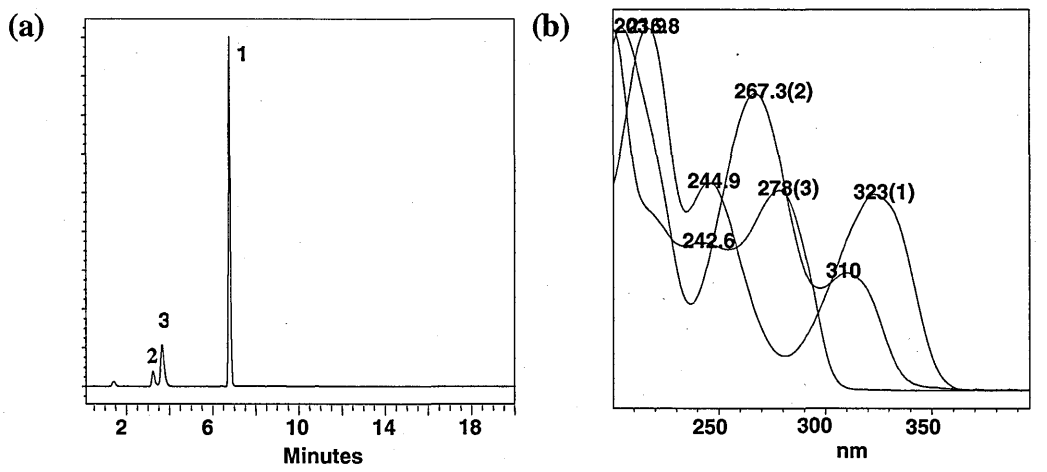


Figure 2.30 (a) Reaction of T^{TRI} & cysteine (peak 2 appeared for a very short time at 5°C) (b) UV λ_{max} of the peaks

The synthesised **20** was purified by silica gel column chromatography and upon analysing by HPLC shows a single peak as shown in figure *2.28 (a)*, which has UV λ_{max} at 278nm as shown in figure *2.31 (b)*.

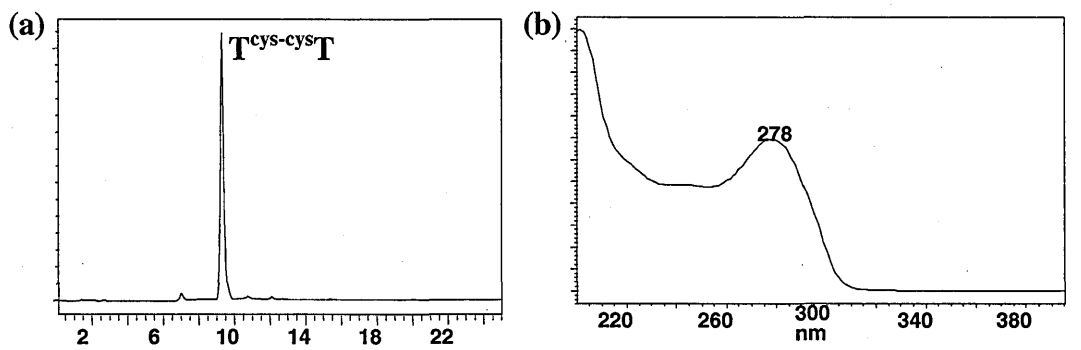


Figure 2.31 (a) 4-cysteinylthymidine (b) UV of 4-cysteinylthymidine

The compound formed was presumed to be **23** rather than **20**. If this is true then the disulphide bonds can be broken by addition of a reagent such as DTT. This reagent is

used in biology laboratories to break the disulphide bonds between proteins. **23** was subjected to react with DTT in water and the reaction was monitored at different time intervals on HPLC.

Reaction of DTT with Peak 4 ($T^{cys\text{-}cys}T$)

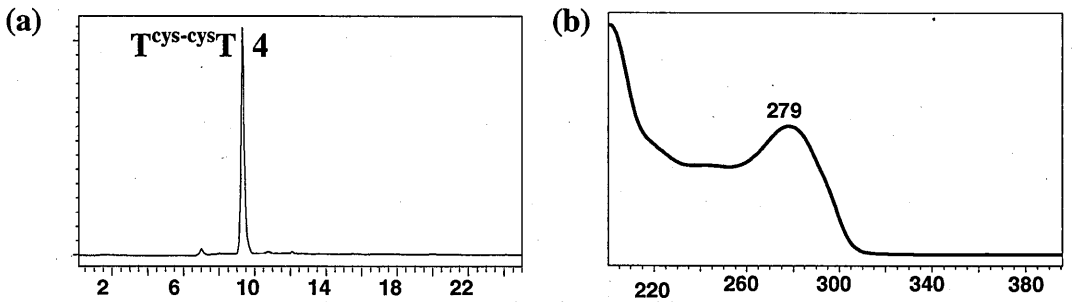


Figure2.32 (a) In the above profile DTT was not added yet.

(b) UV of peak 4 ($T^{cys\text{-}cys}$)

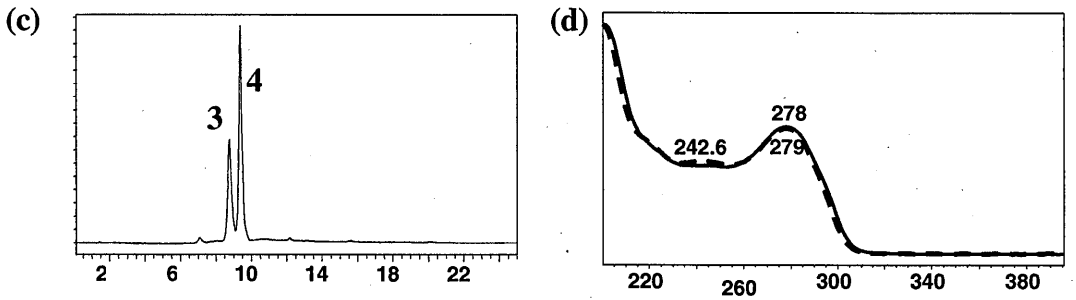


Figure 2.32 (c) DTT was added and injected into HPLC (1hour). Formation of new peak was observed. (d) UV λ_{max} of peak 3& 4.

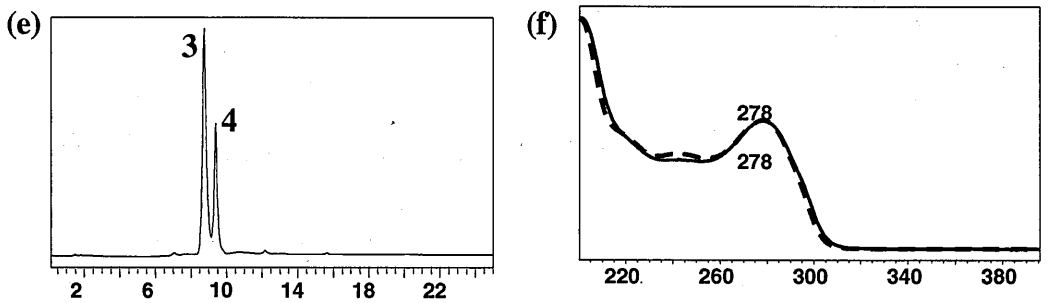


Figure 2.32 (e) after two hours the reaction mixture was again injected into HPLC. New peak (3) is taller than old peak. (f) UV λ_{max} of peak 3& 4.

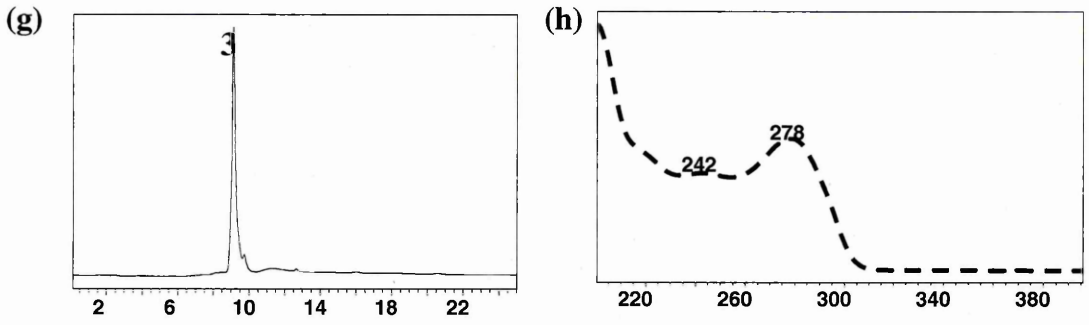
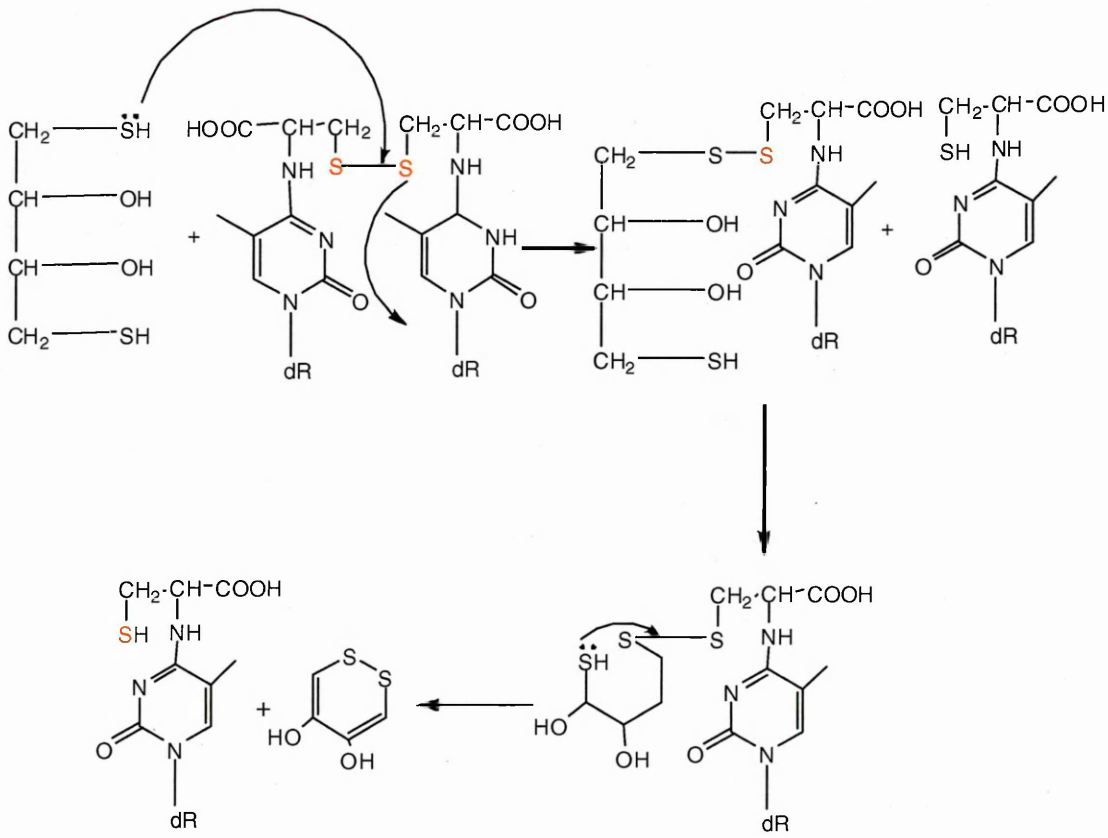


Figure 2.32 (g) Complete conversion of peak 4 to peak 3 was observed. So peak 4 can be confirmed as $T^{cys\text{-}cys}T$. ▬ represents $T^{cys\text{-}cys}T$ (N^4-cysteinylthymidine dimer) --- represents T^{cys} (N^4-cysteinylthymidine)

So Peak 4 was converted to a new peak which had the same retention time and UV as that of peak 3 under the same conditions. This proves that peak 4 is formed from 3 and is dimer of N^4-cysteinylthymidine ($T^{cys\text{-}cys}T$).

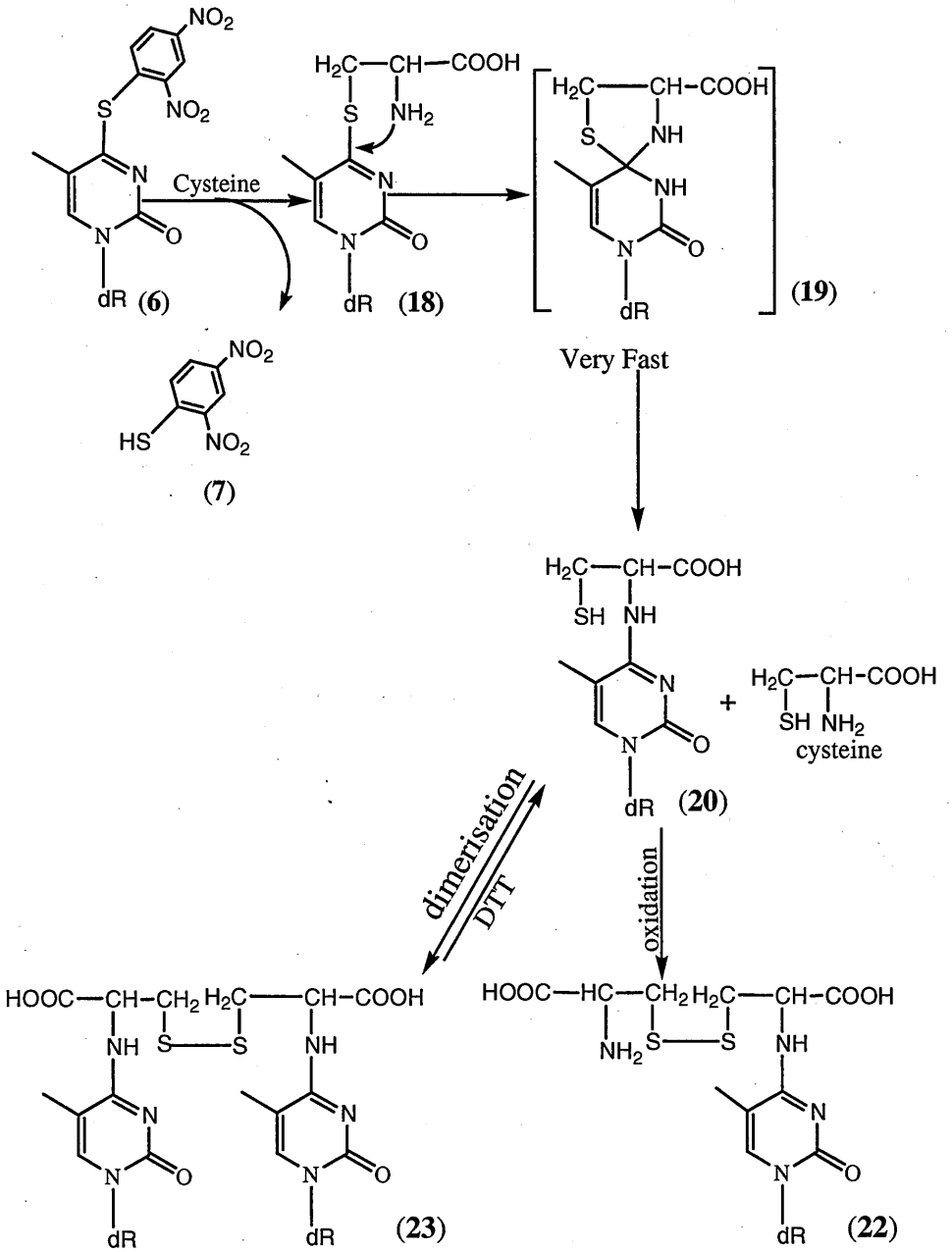
Possible Reaction Mechanism:



Reaction of DTT with $T^{cys\text{-}cys}T$

Thiol groups are susceptible to be oxidised and usually generate disulfides. In this case the free thiol groups of individual molecules of **20** gets oxidised and generates disulfide links among themselves (formation of dimer $T^{cys\text{-}cys}T$). T^{cys} in the presence of free cysteine forms a disulphide link and forms 4-cystinylthymidine ($T^{cys\text{-}cys}$) peak 7.

The entire reaction between T^{SDNP} and cysteine can be represented schematically (see scheme *21*).



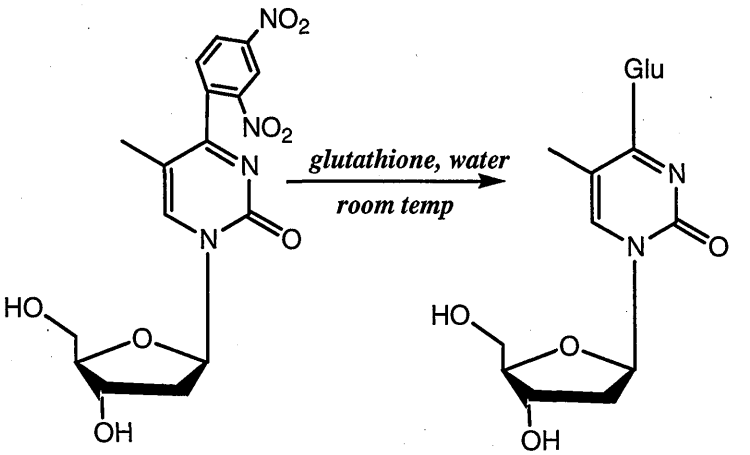
Scheme 21 : Representation of entire reaction between T^{SDNP} and Cysteine.

With all the above evidence it could be concluded that compound **6** reacts with thiol containing cysteine and forms S^4-cysteinylthymidine which rapidly rearranges intra molecularly to form N^4-cysteinylthymidine. The free thiol group in the N4-cysteinylthymidine molecules reacts with either free cysteine to form cystinylthymidine or dimerises.

2.10 Peptide Cross-linking: Reactions with glutathione

Glutathione is widely distributed in animal tissues, plants and micro organisms [110]. It is the most prevalent thiol in the cells and the most abundant low molecular weight peptide [111]. In the cytosol of the cells glutathione reduces disulphide bonds within proteins and protects the cells from reactive oxygen species (ROS). It is an important component of the system that uses reduced pyrimidine nucleotide to provide the cell with its reducing properties, which promotes intracellular formation of cysteine from cystine and thiol forms of proteins. Other important functions of GSH *in vivo* include catalysis, metabolism, and transport. It also participates in the reactions involving synthesis of proteins and nucleic acids. The intracellular level of glutathione is much greater than that of cysteine and serves as a storage for cysteine moieties [111, 112]. Chemically glutathione is a tripeptide consisting of glutamate-cysteine-glycine and is usually denoted by GSH for reduced form and GSSG for oxidised form where two glutathione peptides are joined by a disulphide bond. GSH has two characteristic structural features of γ-glutamate linkage and –SH which are responsible for its intracellular stability and functions [113]. The thiol of glutathione moiety could be useful for site specific cross-linking similar to that of cysteine as seen earlier.

Glutathione reacts with T^{SDNP} and forms 4-glutathionylthymidine (T^{Glu}) as shown in Scheme 22.



(9) (24)

Scheme 22: T^{SDNP} in water reacts with glutathione to form 4-glutathionylthymidine .

The reaction was slow when compared to cysteine and mercaptoethanol. The reaction was monitored by HPLC as shown in Figure 2.33

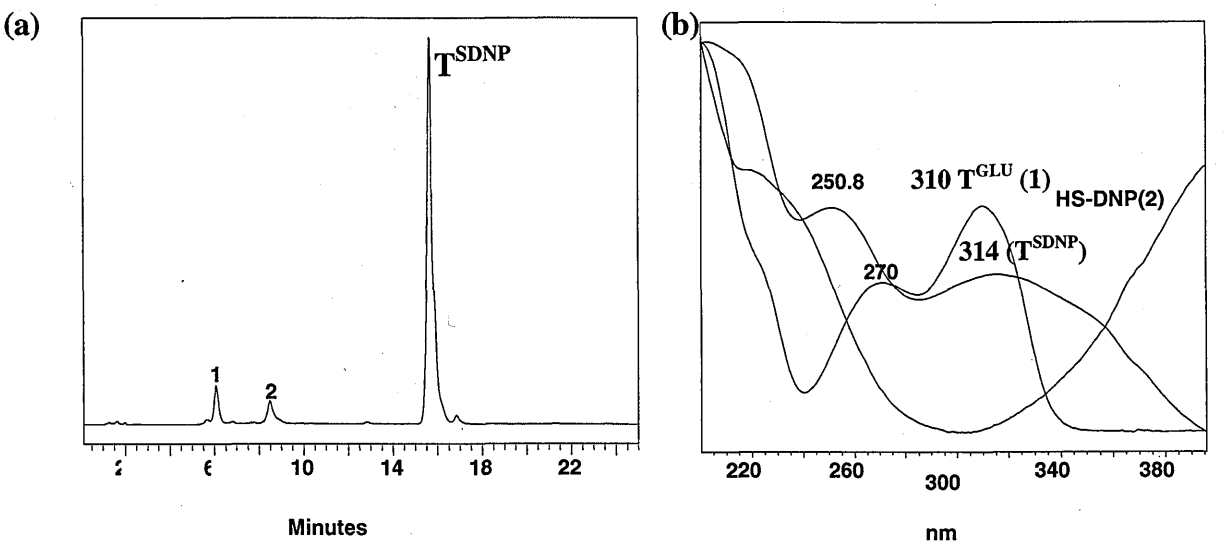
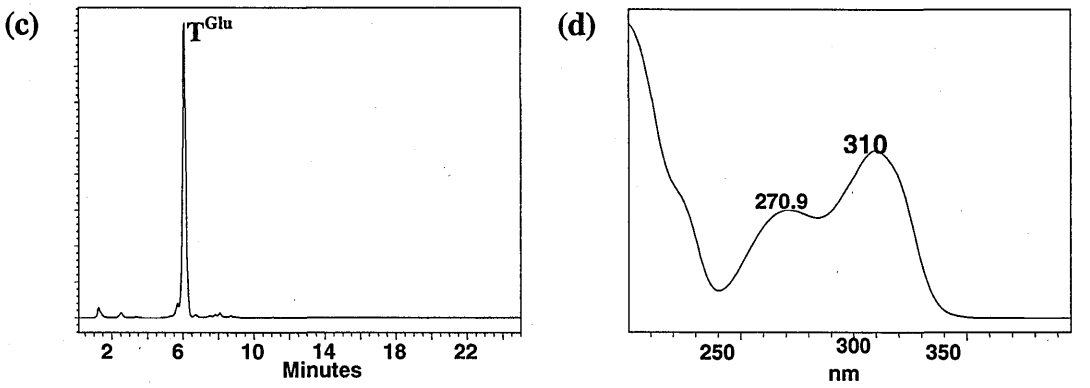


Figure 2.33: (a) glutathione reacts with T^{SDNP} in the presence of water to form T^{GLU}

(b) UV λmax of the individual peaks. λmax = 310 for T^{Glu}.

T^{SDNP} reacts with glutathione in water and forms two new peaks as shown in Figure 2.33. T^{SDNP} was completely converted to peaks 1 and 2 in 24 hours as shown in the following figure 2.33 (c).

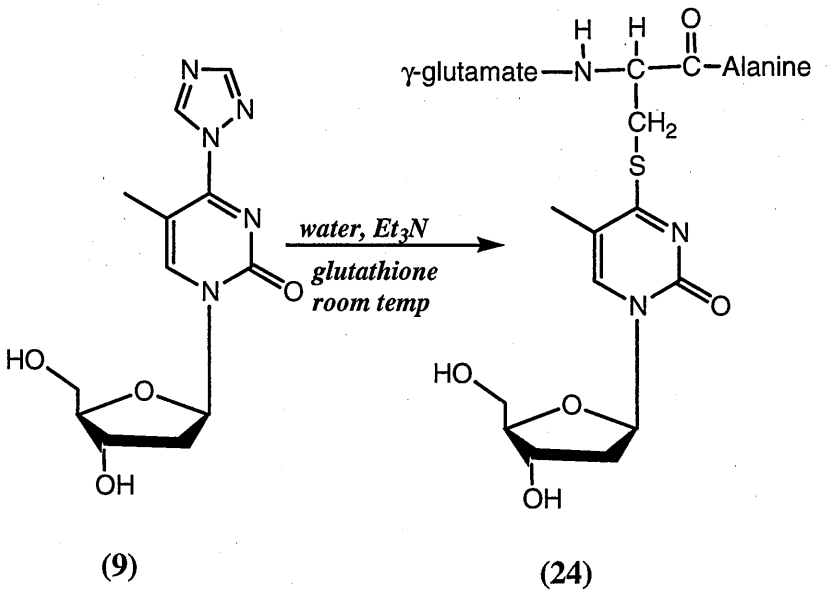


Peak 2 has UV λ_{max} of 406nm and is characterised as HS-DNP as previously. Peak 1 has UV λ_{max} of 310nm. As previously shown in Table 1, S^4-thymidine derivatives have λ_{max} at 310nm. The thiol group of cysteine moiety in glutathione would attack the C4 of T^{SDNP}. Interestingly the product formed with glutathione is S-bonded and remains S-bonded in solution at room temperature. Unlike 4-cysteinylthymidine an N-bonded product was not observed even after a week in the solution. Even though glutathione contains several functional groups, only the thiol of the cysteine moiety attacks the C-4 position of T^{SDNP} to form T^{Glu}. This could be explained by the fact that the adjacent amino group in glutathione is in a peptide amide form and no longer free to attack the C-4 position after the formation of S-bonded product. A standard (**24**) was synthesised for further evidence.

2.11 Synthesis of standard 4-glutathionylthymidine

The same methodology for synthesising 4-cysteinylthymidine and 2-hydroxyethylthiothymidine was employed for synthesising the standard for 4-glutathionylthymidine. This involves the 4-triazolothymidine as the starting material

which reacts with glutathione in the presence of triethylamine to form 4-glutathionyl thymidine **24**.



(9) **(24)**

Scheme 23: 4-triazolothymidine in water reacts with glutathione in the presence of triethylamine at room temperature to form 4-glutathionylthymidine

The purity of the synthesised compound was checked on HPLC which shows a single peak.

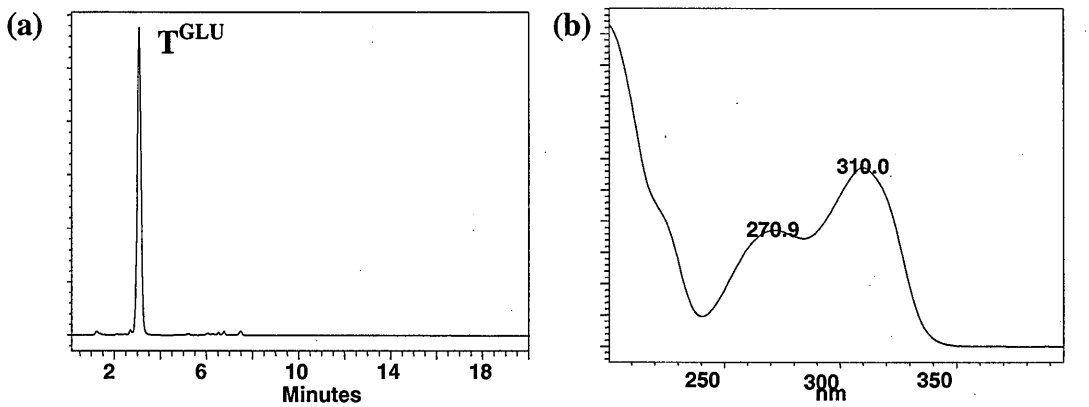


Figure 2.34: (a) Synthesised 4-glutathionylthymidine shows a single peak implying high degree of purity. (b) UV of T^{GLU}

The compound was characterised and confirmed by the data from high resolution mass spectrometry *m/z* 532.1705 ([M^++1]), which was then co-injected with the reaction mixture of T^{SDNP} and glutathione (see figure *2.34*).

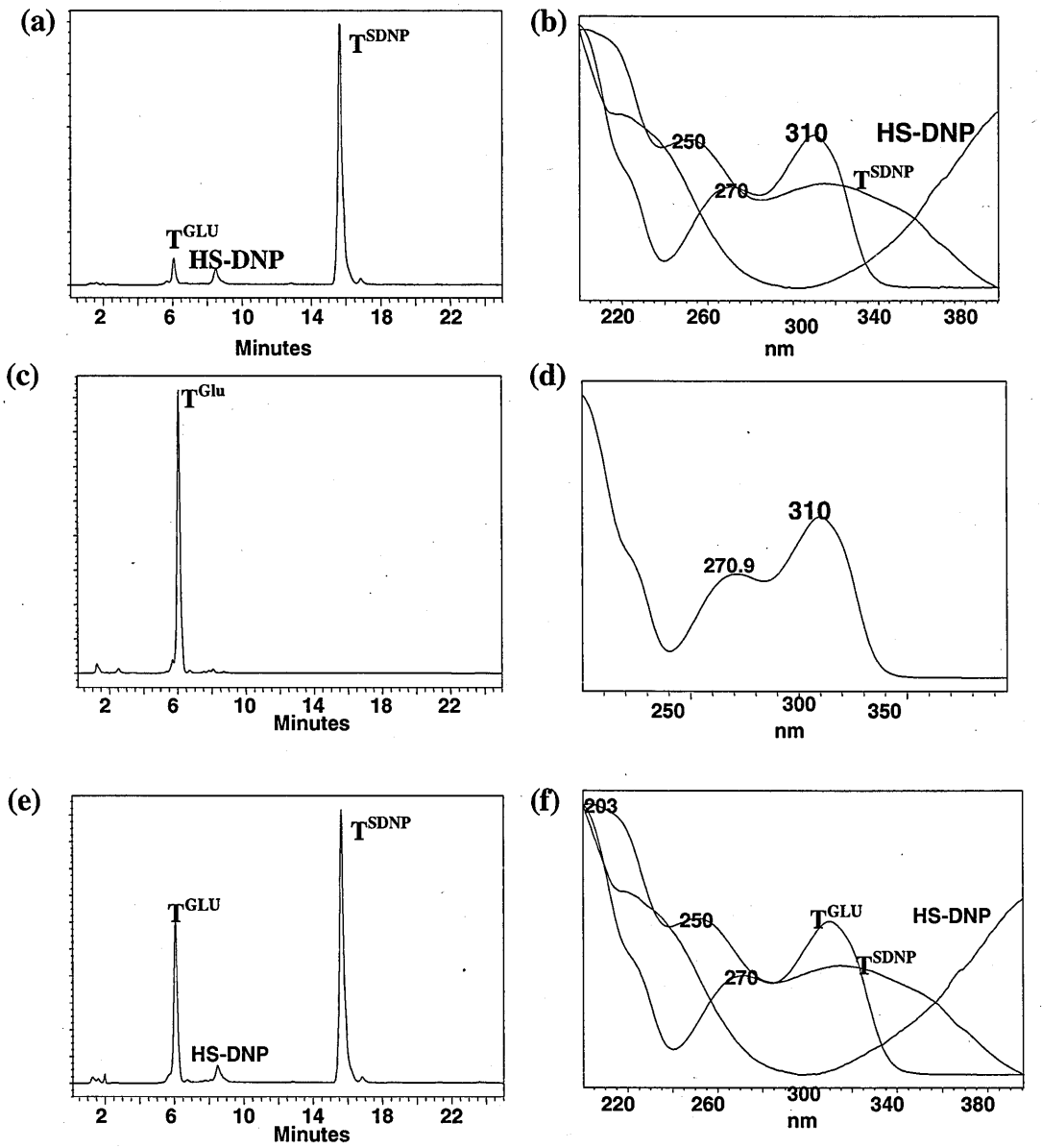


Figure 2.35 (a) Reaction of T^{SDNP} with glutathione. (c) Standard T^{GLU}. (e) Co-injection of (a) and (c) shows increase in the peak height of T^{GLU} in relation to the adjacent peaks.

This co-injection experiment (Figure 2.35) proves that the product formed from the reaction between T^{SDNP} and glutathione was T^{GLU}.

In summary, from the results so far it could be concluded that 4-thiothymidine can be activated by various reagents such as FDNB/ClDNB to form a good leaving group – 4-S-(2,4-dinitrophenyl) - which can be specifically substituted by various thio-nucleophiles. The thio-nucleophiles could be either chemical reagents such as mercaptoethanol, ethanethiol or unique biological molecules such as cysteine and glutathione.

These results and methodology could be extended to various other thiopurine or thiopyrimidine analogues and also to the DNA containing thionucleosides.

CHAPTER 3

Synthesis and Reactions of 4-Thio-5-bromodeoxyuridine

3 Synthesis and reactions of 4-thio-5-bromodeoxyuridine

3.1 Introduction

Bromodeoxyuridine (BrdU) is a thymidine analogue with a bromine substituent at position 5. BrdU can be incorporated into DNA during synthesis and is a very useful biochemical tool that has also attracted interest as a chemotherapeutic agent in the treatment of prostrate cancer. Monoclonal antibodies that recognise BrdU are used to identify cells that have incorporated BrdU and are used to detect and measure cell proliferation [76]. BrdU is a potent sensitizer cancerous cells when compared to 5-fluorouracil (5-FU) in the treatment of some forms of cancers.

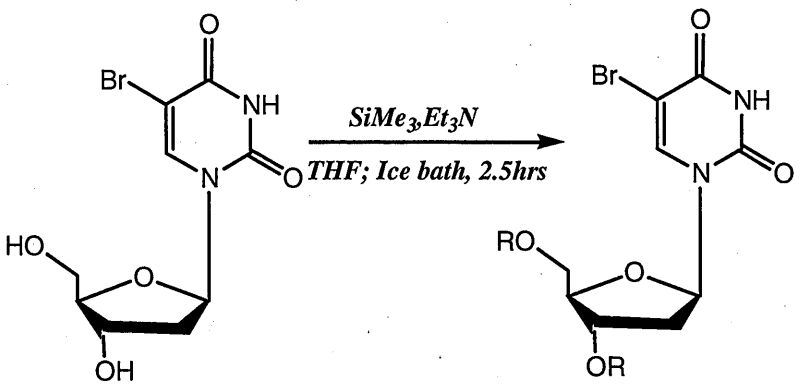
In the quest to develop new novel synthetic drugs against cancer, 4-thio-5-bromo-2'-deoxyuridine (S^4-BrdU) was recently synthesised in our group as a reagent for UVA induced cell killing [76]. The advantage of S^4-BrdU over 4-thiothymidine was that it could be recognised by the available monoclonal antibodies used against DNA containing BrdU [76]. The cross-recognition was ascribed to the presence of the large bromine atom in S^4-BrdU as a distinctive structural feature compared to the relatively small effect of substituting sulphur for oxygen.

An important property similar to 4-thiothymidine was that S^4-BrdU was not toxic to cells over a range of concentrations [76]. The very promising results for cross-linking with 4-thiothymidine reported in the previous chapter prompted us to explore the potential of S^4-BrdU for site-specific cross-linking. Xu *et al*[76] first reported the synthesis of S^4-BrdU and its key properties. Synthesis of S^4-BrdU is similar to that of 4-thiothymidine and involves the 4 steps as shown in Scheme 3.1 (a) to 3.1 (d).

3.2 Synthesis of S^4-BrdU

Step-1

The first step involves the protection of 3' and 5' OH groups of sugar with trimethylsily groups as shown in Scheme 3.1 (a). 5-Bromodeoxyuridine (**25**) was suspended in THF and reacted with trimethyl chlorosilane in the presence of triethyl amine to give 3`, 5`-O-bis-(trimethylsilyl)-5-bromodeoxyuridine (**26**).



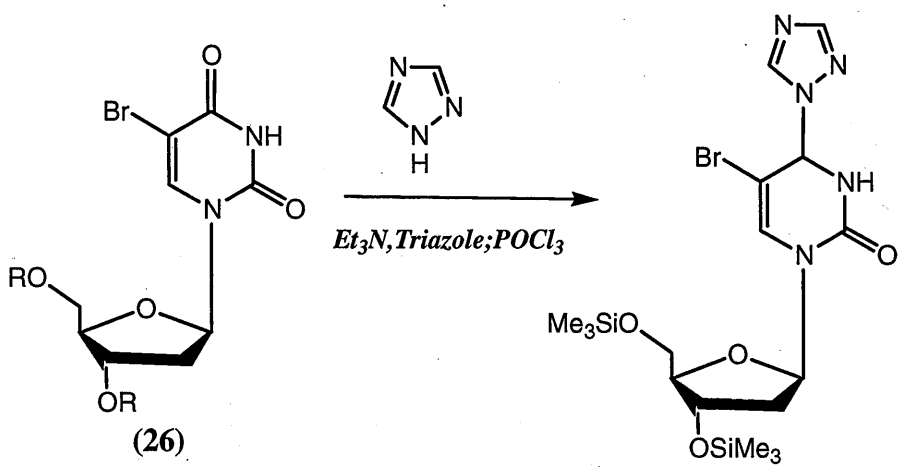
(25)
5-bromodeoxyuridine

(26)
3',5'-O-bis-(trimethylsilyl)-5-bromo deoxyridine.

Scheme 3.1 (a) Protection if 3' and 5' OH of thymidine

Step-2

26 in acetonitrile was treated with 1,2,4-1H triazole in the presence of triethylamine and phosphorous oxychloride to give 4-triazolo-3`,5`-O-bis-(trimethylsilyl)-5-bromodeoxyuridine as shown in Scheme 3.1 (b).



(26) **(27)**

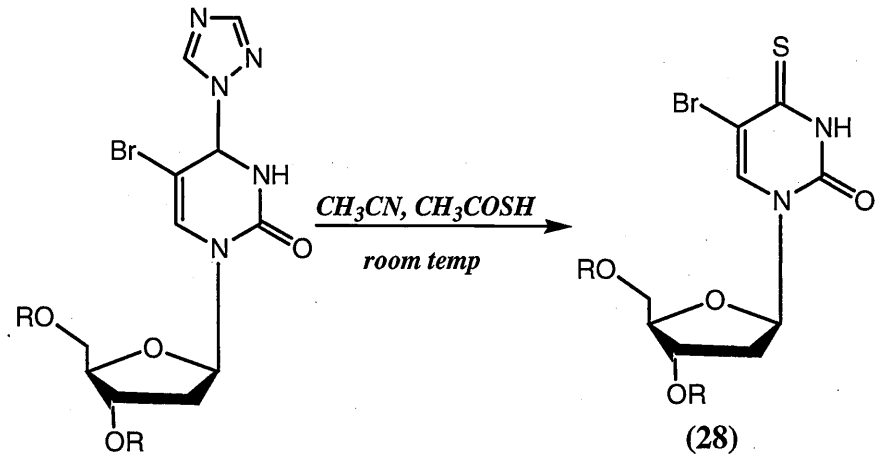
3',5'-O-bis-(trimethylsilyl)-5-bromo deoxyridine. **4-triazolo-3',5'-O-bis-(trimethylsilyl)-5-bromodeoxyridine.**

Scheme 3.1(b) Triazolation of C4 of thymidine

Phosphorous oxychloride and 1,2,4-(1H)-triazole are added to **26** in acetonitrile. Triethlylamine is added in 2:1 molecular ration in the beginning of the reaction and is not only used to neutralise the formed HCl but also abstract a proton from 1-N of triazole.

Step-3

27 in acetonitrile was treated with thiolacetic acid to give 4-thio-3`,5`-O-bis-(trimethylsilyl)-5-bromodeoxyuridine.



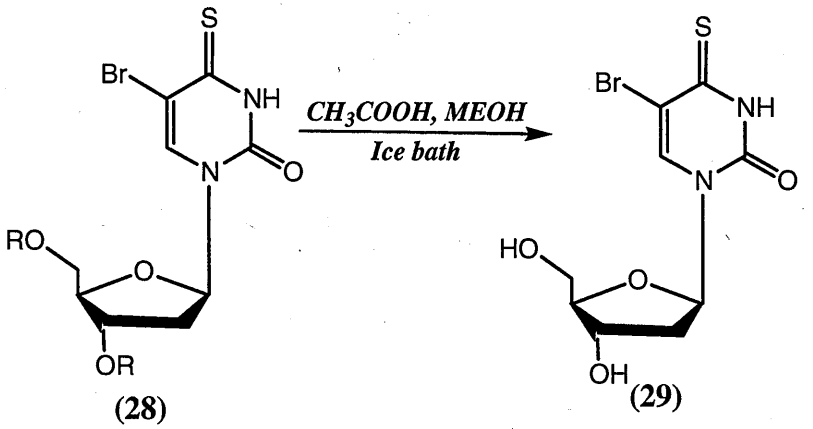
(27) **(28)**

4-triazolo-3',5'-O-bis-(trimethylsilyl)-5-bromodeoxyridine. **4-thio-3',5'-O-bis-(trimethylsilyl) -5-bromodeoxyridine.**

Scheme 3.1 (c) thiol group formation at C4

Step-4

The trimethylsilyl groups hydrolyse in the presence of acid and form 4-thio-5-bromodeoxyruidine.



(28)
4-thio-3',5'-O-bis-(trimethylsilyl) -5-bromodeoxyridine.

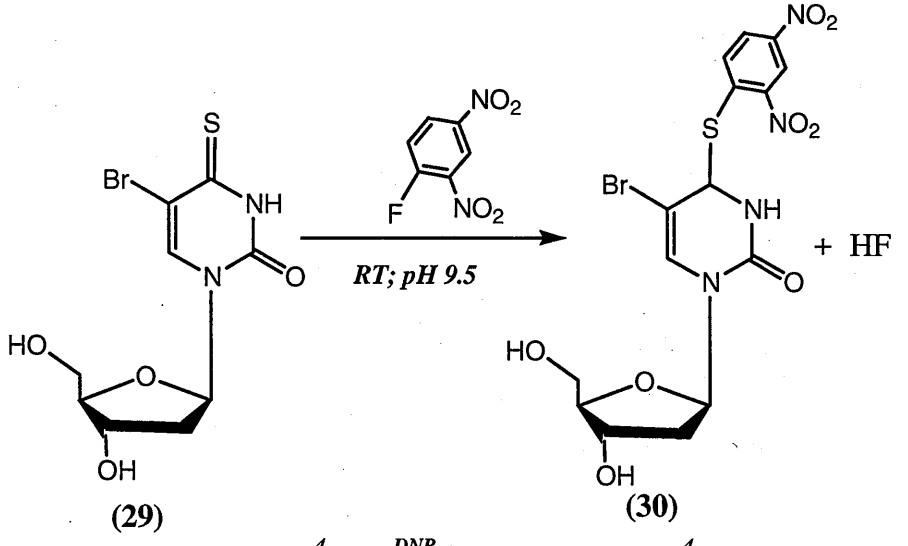
(29)
4-thio-5-bromodeoxyuridine

Scheme 3.1 (d) formation of 4-thio-5-bromodeoxyuridine

3.3 Modification of S^4-BrdU

A similar protocol to 4-thiothymidine was followed for the modification of 4-thio-5-bromodeoxyuridine (S^4-BrdU). As shown earlier for 4-thiothymidine, the chosen reagent for the activation of S^4-BrdU was 2,4-dinitrofluorobenzene (FDNB).

FDNB reacts with **29** in the presence of phosphate buffer pH 9.5 to form S^4-2,4 dinitrophenyl-5-bromodeoxyuridine (S^4-$BrdU^{DNP}$) as shown in Scheme 3.2.



(29) **(30)**

Scheme 3.2: Formation of S^4-$BrdU^{DNP}$ from the reaction of S^4-BrdU and FDNB

The reaction was monitored on HPLC as shown in figure 3.1

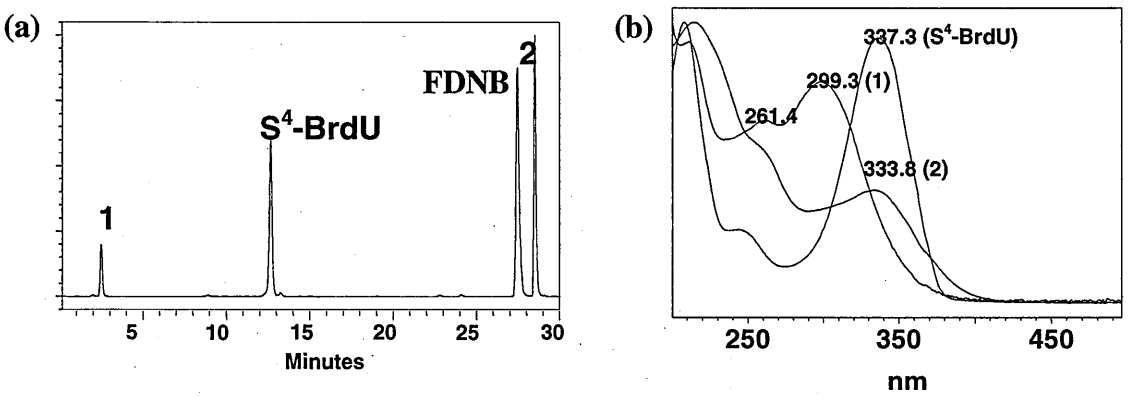


Figure 3.1: (a) Reaction of S^4-BrdU with FDNB in phosphate buffer (b) UV λ_{max}

Two new peaks were observed. Peak 1 has retention time at 2.5 minutes with a UV λ_{max} 299.3nm and peak 2 was eluted at 29 minutes with a λ_{max} of 333.8nm.

A blank was run to identify the above observed peaks.

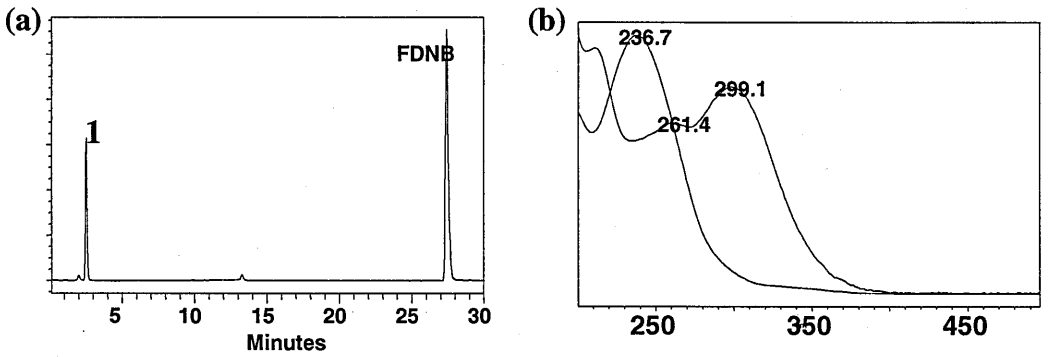


Figure 3.2 (a) FDNB and phosphate (pH 8.5). (b) UV λ_{max} of the peaks

When the phosphate buffer (pH 9.5) was added to the reagent FDNB a new peak (1) was observed. This new peak has the same retention time and UV as peak (1) in Figure 3.1 (a) of the reaction mixture. This confirms that the peak 1 in figures 3.1 (a) and 3.2 (a) are the same.

Blank reaction with S^4-BrdU and phosphate buffer was monitored by HPLC

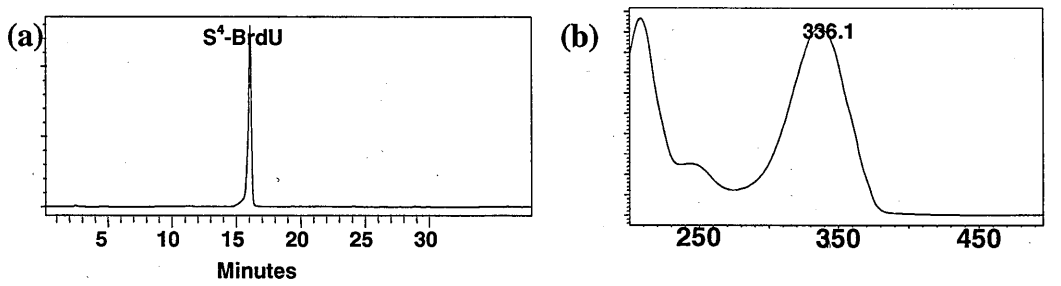


Figure 3.3 (a) S^4-BrdU was added to phosphate buffer at pH 9.5 and monitored on HPLC (b) UV λ_{max} of peak of S^4-BrdU

As monitored above on the HPLC new peaks were not observed. So peak 1 was formed from the reagent FDNB and not significant to study further. Peak 2 has UV λ_{max} at 333.8nm which is typical for S^4-$BrdU^{DNP}$.

The peak 2 of S^4-2,4-$BrdU^{DNP}$ was isolated as shown in the figure 3.4 (a) and the UV λ_{max} was measured as in 3.4 (b). This isolated peak was used for further reactions with thiols.

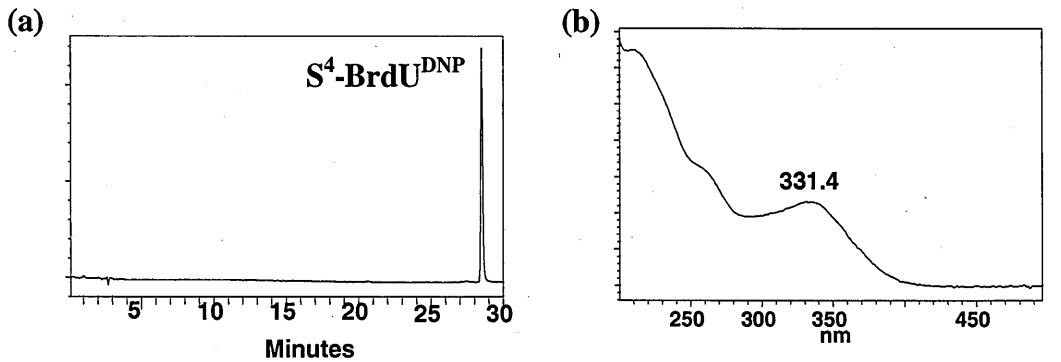
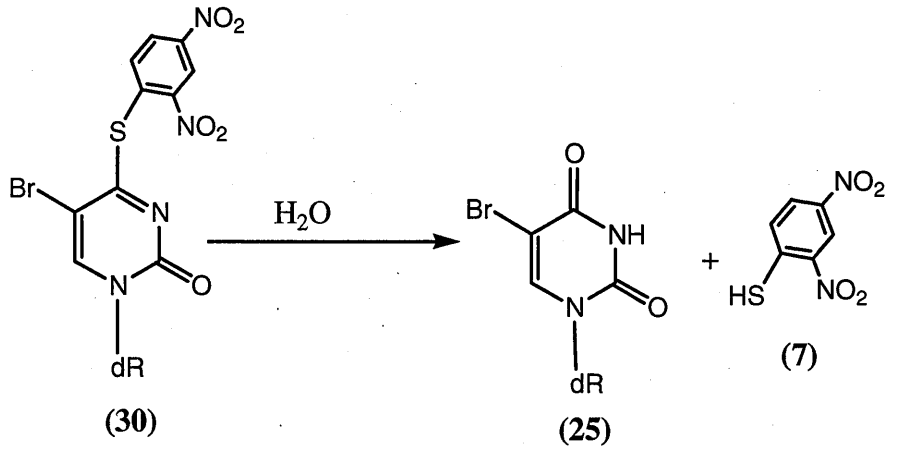


Figure 3.4 (a) Isolated peak of S^4-$BrdU^{DNP}$ (b) λ_{max} of S^4-$BrdU^{DNP}$

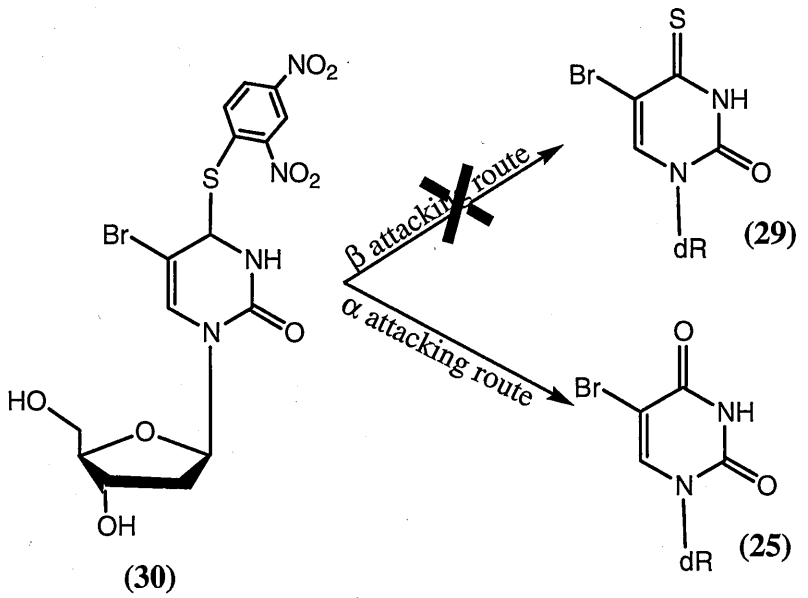
3.4 Reaction of S^4-$BrdU^{DNP}$ with various nucleophiles:

30 in water for 7 days hydrolyse and form **25** and **7** as shown in scheme 3.5.



Scheme 3.5 Hydrolysis of S^4-$BrdU^{DNP}$

Only compound **25** was observed on hydrolysis. Compound **29** was not observed. This is consistent with the data observed for compound **6**. So it could be concluded that dinitrothiopyrimidines undergo only nucleophilic attack at C-4 (α-attacking route) and not C-1 of the aromatic ring (β-attacking route) as shown in scheme 3.6. This is different for dinitrothiopurines as reported by Xu [94] as shown in scheme 8.



Scheme 3.6 attacking route of S^4-$BrdU^{DNP}$

Compound **30** was subjected to react with various thionucleophiles such as sodium sulphide and mercaptoethanol to confirm the mechanism in scheme 3.6

3.5 Reaction of S^4-2,4-$BrdU^{DNP}$ with sodium sulphide:

30 was treated with sodium sulphide and injected into HPLC. The observations were as follows.

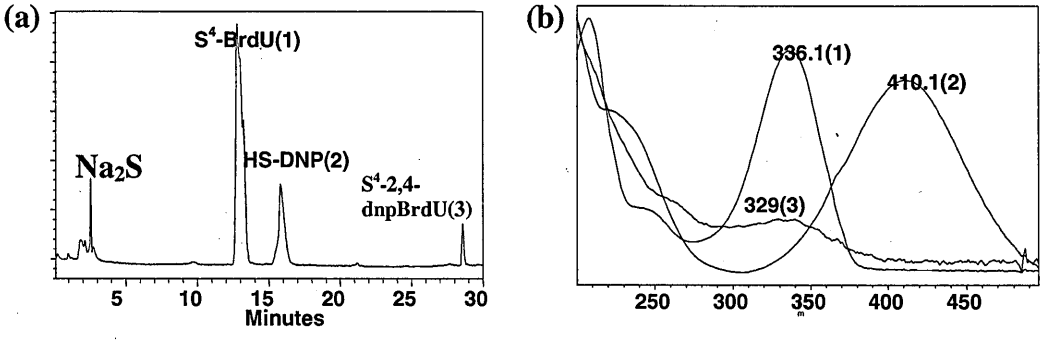


Figure 3.4: (a) reaction of S^4-2,4$BrdU^{DNP}$ with Na_2S (b) UV of the peaks

Sodium sulphide reacts with **30** and forms **29** and **7**. In this reaction **25** was not observed in the above profile. The sulphur in **29** is formed from sodium sulphide. The sulphur atom from **30** transferred to compound **7**. Standards of compound **25** and compound **29** were injected to compare the retention times.

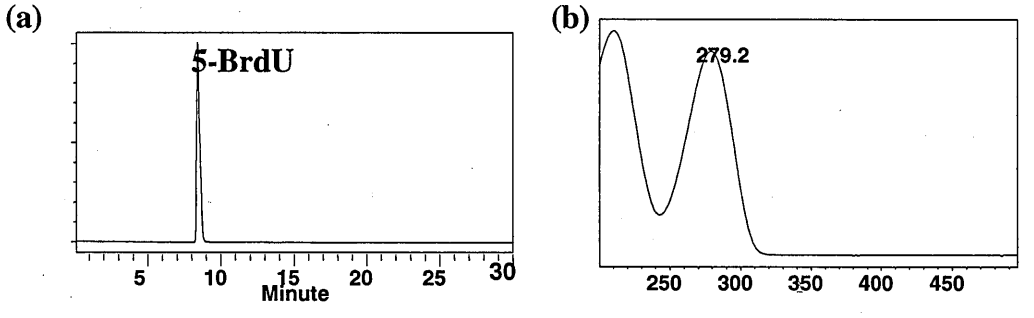


Figure 3.5: standard of 5-BrdU (b) UV of 5-BrdU

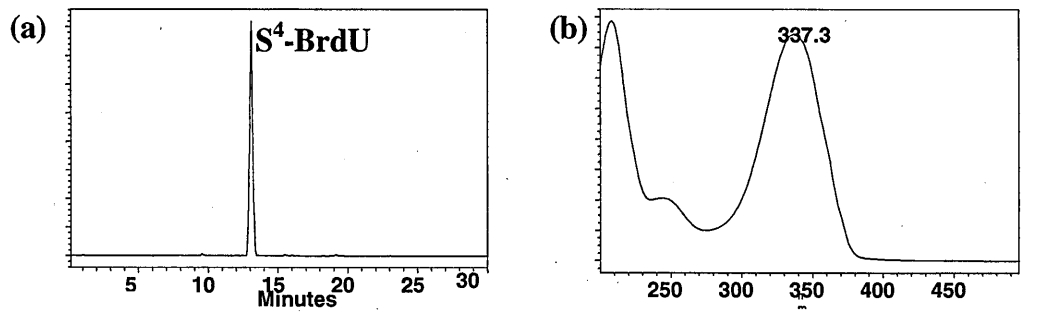


Figure 3.6: standard of S^4-BrdU (b) UV of S^4-BrdU

The peaks from the reaction mixture in Figure 3.4a and the standard (S4-BrdU) have similar retention times (13 minutes). So a co-injection was performed to confirm the product.

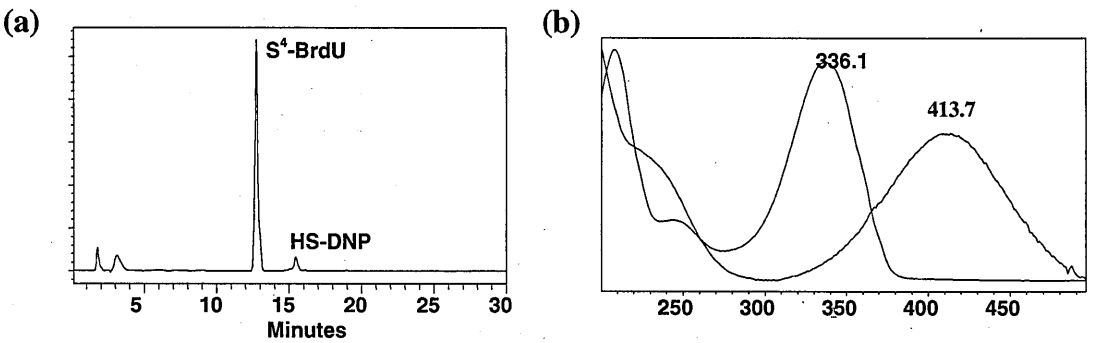
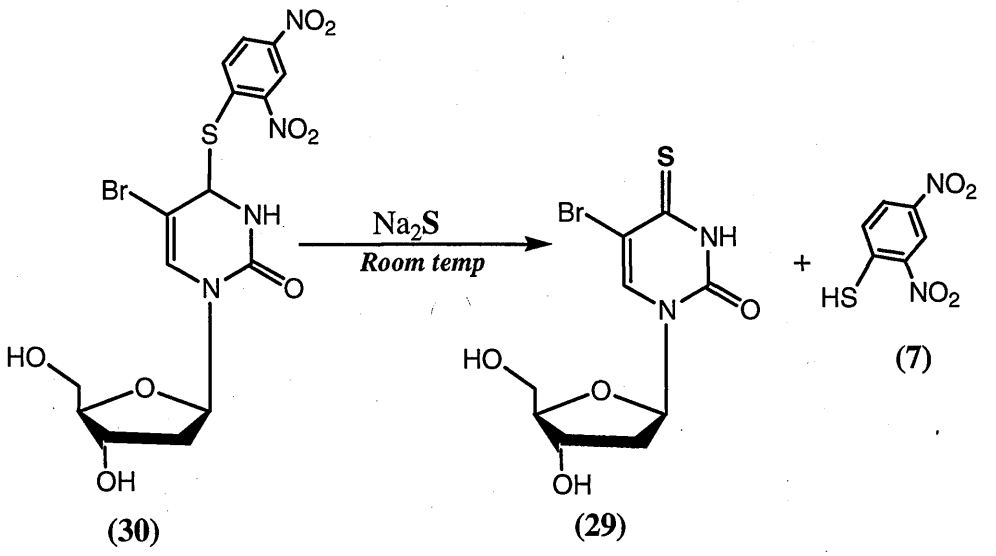


Figure 3.7: Co-injection of S^4-BrdU with the reaction mixture of S^4-$BrdU^{DNP}$ with Na_2S

So the co-injection from Figure 3.7 confirms that the product formed from the reaction of **30** with sodium sulphide was that of **29**. Since 5-BrdU peak was not observed, it could be confirmed that like the compound **6,** compound **30** also undergoes thiol substitution as shown in Scheme 3.5.



Scheme 3.5: Sodium sulfide reacts with S^4-$BrdU^{DNP}$ to form S^4-BrdU

3.6 Reaction of S^4-DNPBrdU with various thiols:

3.6 (a) Reaction with mercaptoethanol

To show that compound **30** could be used as a good intermediate for cross-linking it was first treated with mercaptoethanol at room temperature and the reaction was followed by HPLC. The observations were as follows:

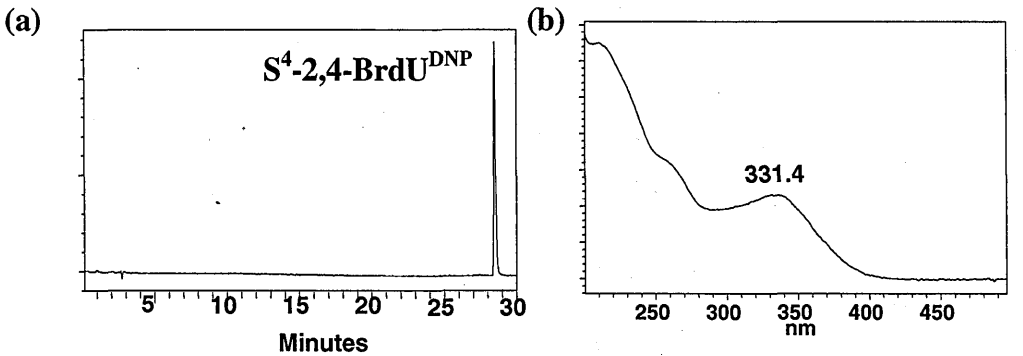


Figure 3.8: S^4-2,4-$BrdU^{DNP}$ (b) UV λ_{max} of S^4-2,4-$BrdU^{DNP}$

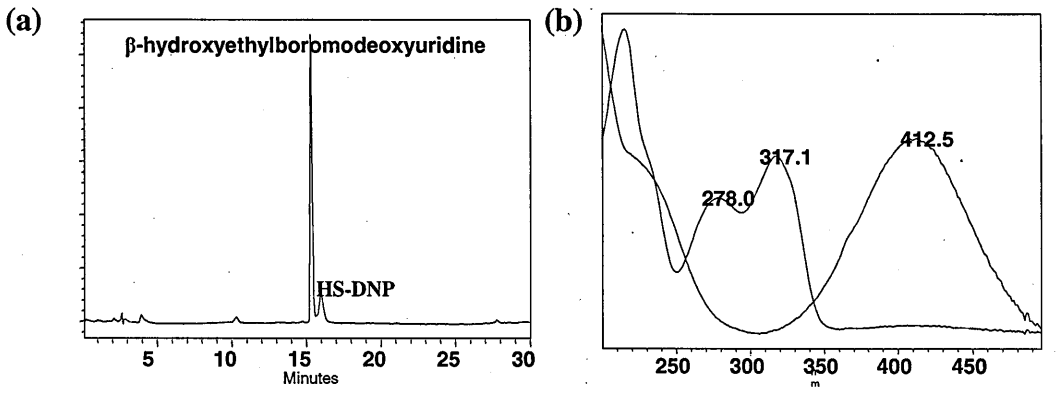
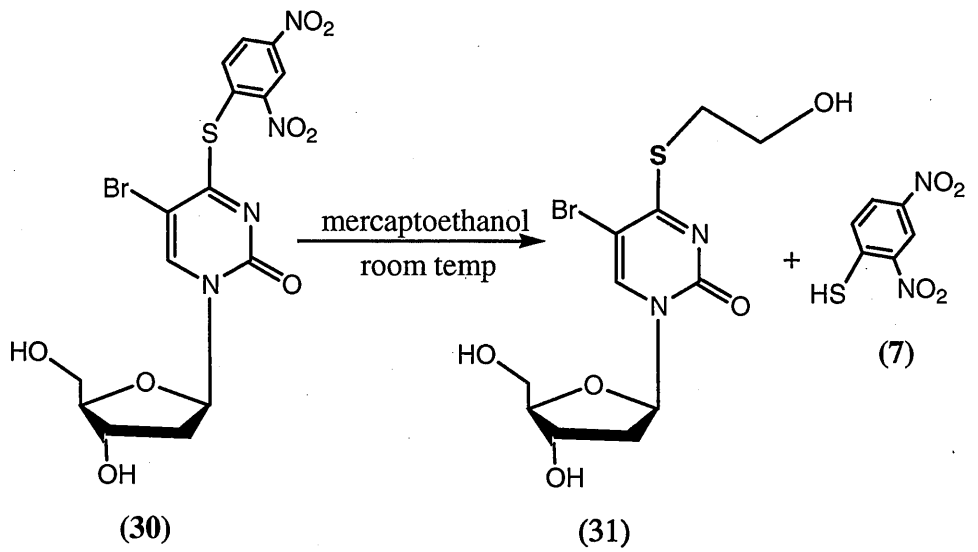


Figure 3.9 reaction of S^4-2,4BrdU^{DNP} with mercaptoethanol

Compound **30** was first injected into the HPLC on its own as shown in figure 3.8. Then an equivalent amount of meracaptoethanol was added to the same vial and immediately injected into HPLC. The compound **30** has completely changed to a major new peak with UV λ_{max} of 317nm and minor peak at 412.5nm as shown in figure 3.9 . This new peak can be tentatively assigned as the compound **31**. As per our earlier discussion the λ_{max} for **8** was 309nm and all the S^4-bonded thymidine compounds would have UV λ_{max} of more than 300nm. So compound **30** can react with thionucleophile such as mercaptoethanol and form 2-hydroxyethylthiobromodeoxyuridine (**31**) and dinitrothiophenol (**7**) as shown in Scheme 3.6.



Scheme 3.6: Mercaptoethanol reacts with S^4-BrdU^{DNP} to form 2-hydroxyethylthiobromodeoxyuridine

3.7 Reaction of S^4-$BrdU^{DNP}$ with cysteine

30 was treated with cysteine at room temperature and the reaction was monitored on HPLC. The observations were as follows:

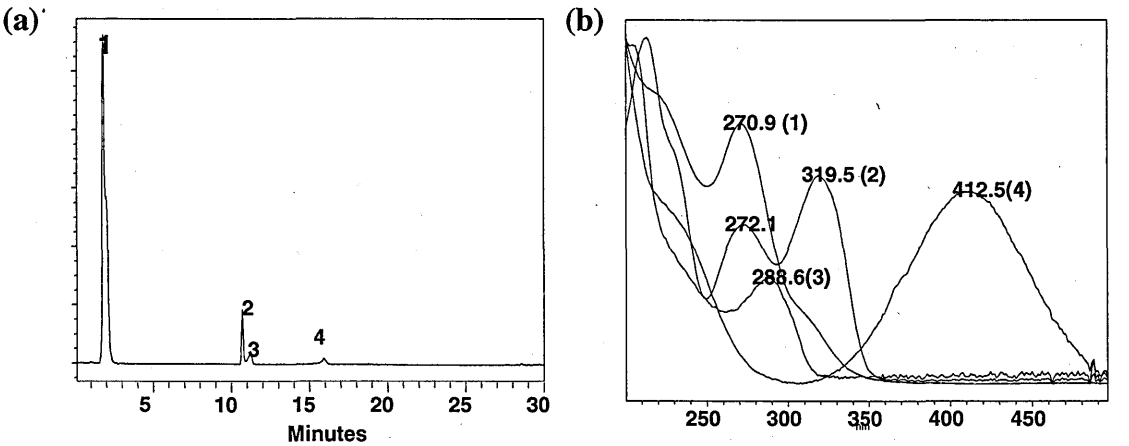


Figure 3.10: Immediate injection of reaction of 30 with cysteine

Peak 1 can be assigned as the reagent cysteine. Peak 2 has UV λ_{max} of 319.5nm. As discussed earlier all the S^4-bonded pyrimidines have the UV λ_{max} at more than 300nm. So, peak 2 can be tentatively assigned as S^4-cysteinyl-5-bromodeoxyuridine. Peak 3 has UV λ_{max} at 288.6nm which is characteristic of N^4-bonded pyrimidine derivatives. So peak 3 can be tentatively assigned as N^4-cysteinyl-5-bromodeoxyuridine. This peak should increase over the time if compound **30** undergoes similar substitution with cysteine. So the same reaction mixture was injected into HPLC and monitored after 4-hours and the observations were as follows:

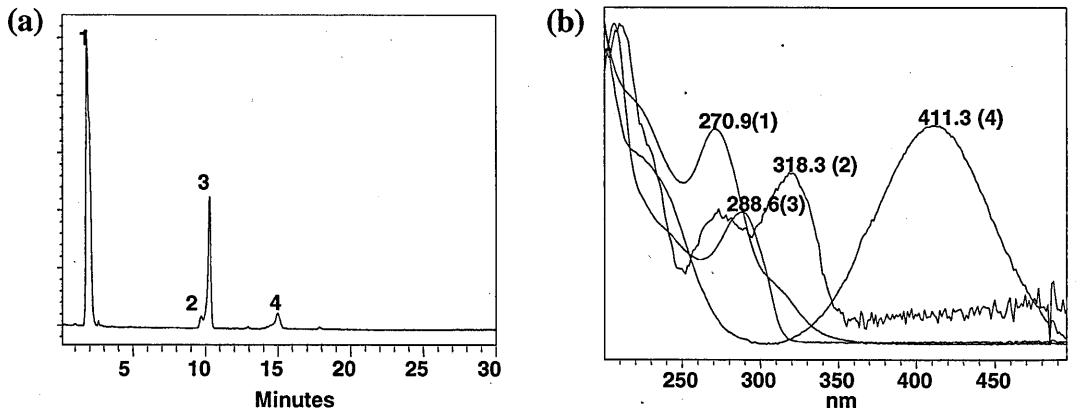
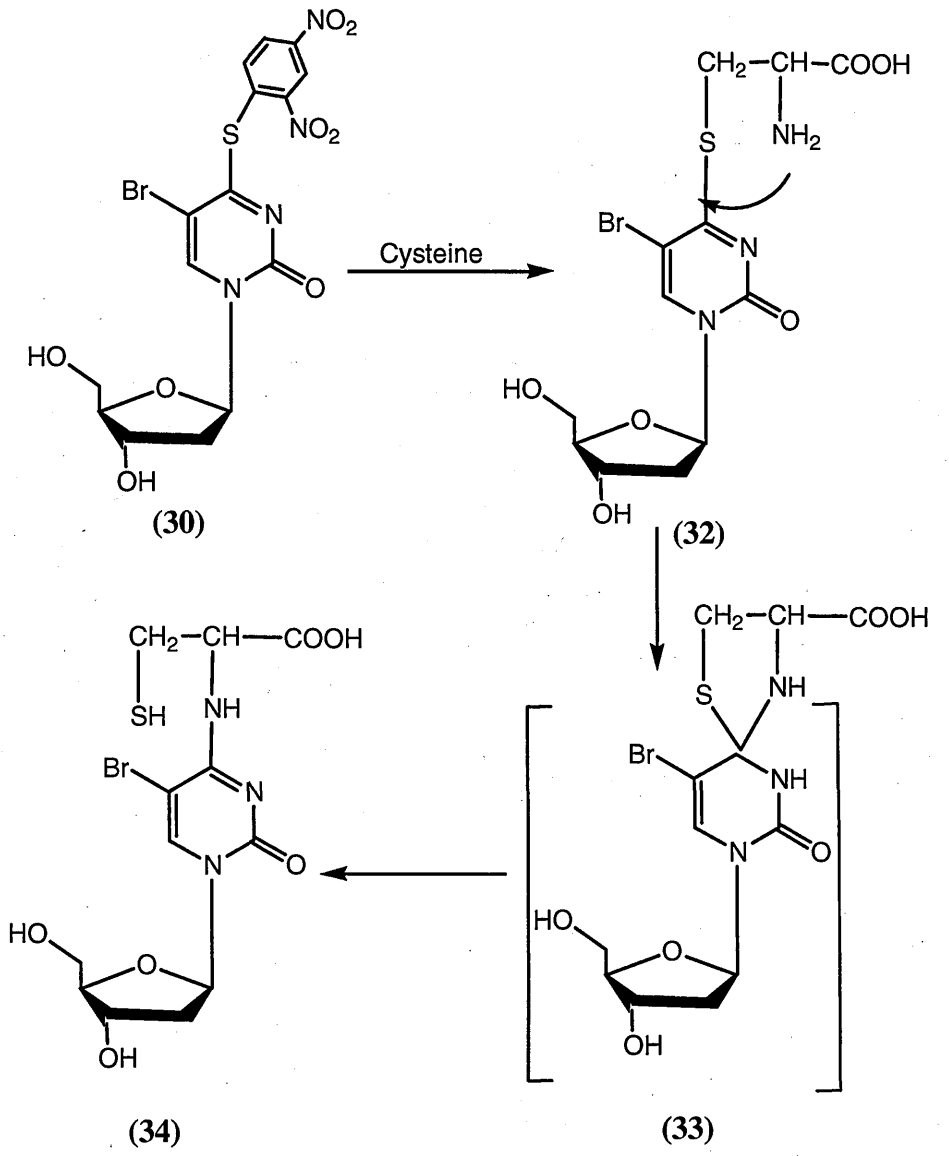


Figure 3.11: after 4 hours peak 2was converted to peak 3

(Conversion of S-bonded 4-cysteinyl-BrdU to N-bonded product)

As expected peak 2 was almost completely converted into peak 3 after 4hours (as seen in figure 3.11) and this would support our proposed mechanism that first the thiol of cysteine would attack followed by ring rearrangement to a stable N-bonded 4-cysteinyl-5-bromodeoxyuridine.

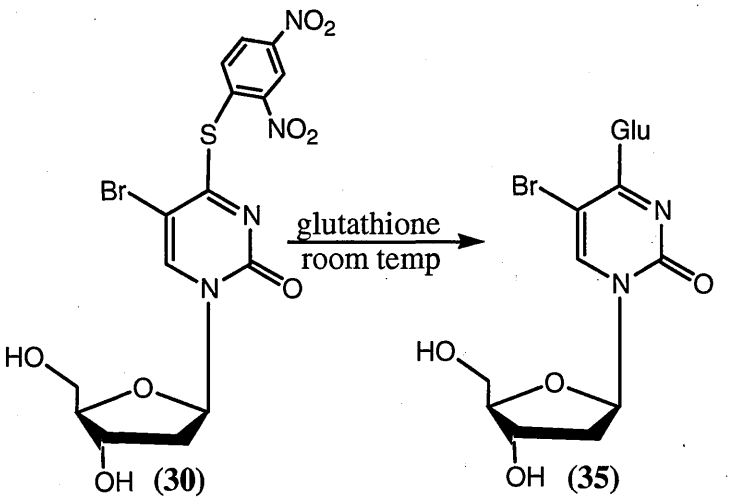
We propose the reaction mechanism as similar to that of previously described for formation of **20**. The excerpts of the reaction mechanism for the formation of 4-cysteinyl-5-bromodeoxyuridine were, first the thiol group of cysteine molecule would attack at C-4 position of **30** and forms an S^4-cysteinyl-5-bromodeoxyurine which would then be attacked by the adjacent amino group in the same cysteine molecule to form an N^4-cysteinyl-5-bromodeoxyuridine.



Scheme 3.7 :Conversion of S^4-BrdU^{DNP} to N^4-cysteinyl-5-BrdU

3.8 Reaction with glutathione

Glutathione reacts with S^4-DNPBrdU to form 4-glutathionyl-5-bromodeoxyuridine. The reaction was very slow when compared to that of other thionucleophiles such as sodium sulphide, mercaptoethanol and cysteine. This could be due to the steric bulk of the glutathione molecule making the thiol in glutathione less reactive.



Scheme3.8: S^4-$BrdU^{DNP}$ in water reacts with glutathione to form 4-glutathionyl-5-bromodeoxyuridine

Glutathione was added to **30** in water and injected into HPLC as shown in figure 3.12. Two new peaks were observed. Peak 1 is that of compound **30**, peak 2 has UV λ_{max} at 319nm. Peak 3 has UV λ_{max} at 406.5nm which is consistent with that of dinitrobenzenethiol (**7**) as previously discussed.

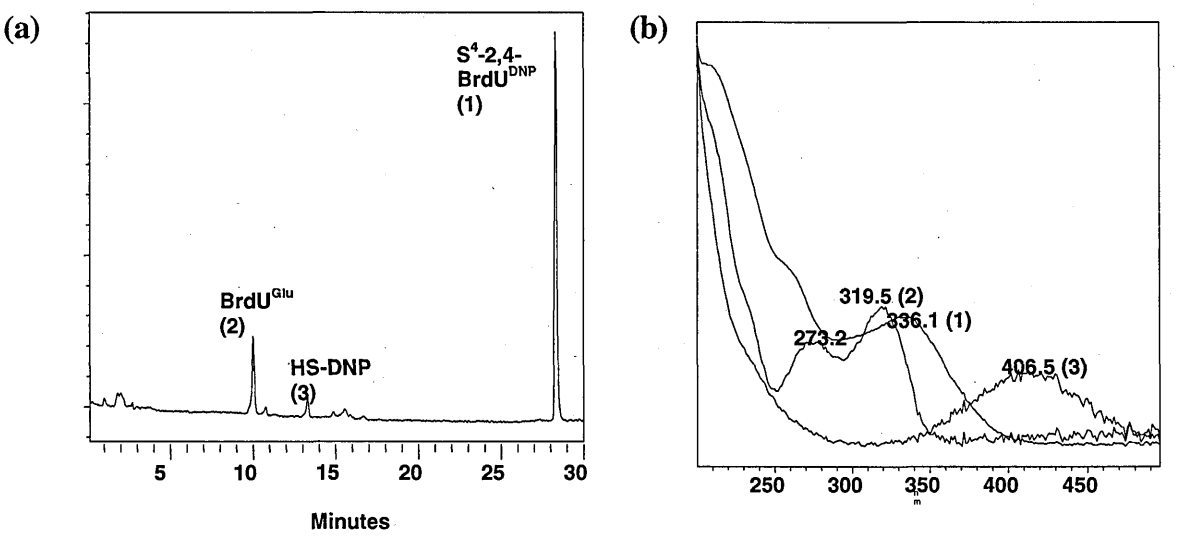


Figure 3.12 (a) reaction of S^4-2,4-dnpBrdU with glutathione the reaction is slow when compared with the reaction with formerly used thiol reagents under the same conditions(b) UV λ_{max} of the peaks

The reaction was monitored at different time intervals (data not shown) and when the reaction was left overnight the starting material (S^4-DNPBrdU) was completely converted to new peak of 4-glutathionylbromodeoxyuridine ($BrdU^{Glu}$).

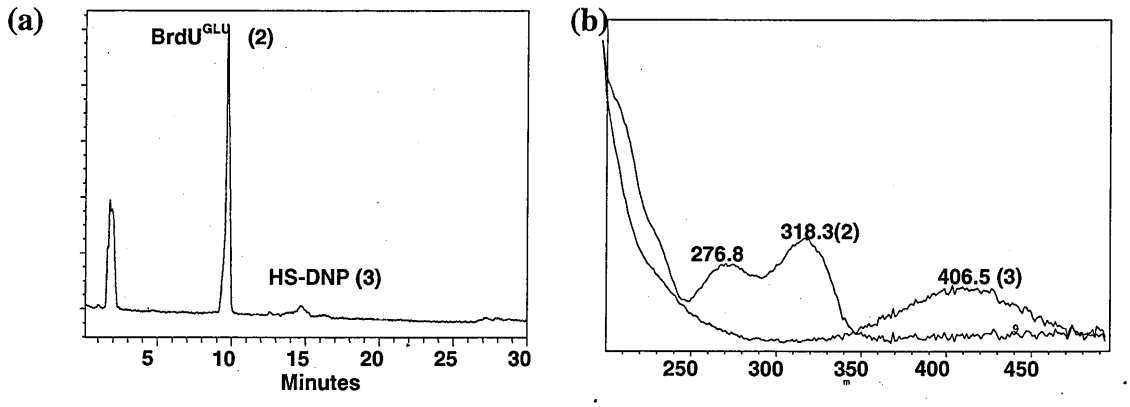


Figure 3.13: (a) the same reaction mixtures from figure 3.12 were re-injected after leaving for overnight for the reaction to complete (starting material completely converted to product) (b) UV λ_{max} of the peaks were shown

3.9 Conclusions

S^4-DNPBrdU undergoes the same kind of nucleophilic substitution as that of S^4-DNPthiothymidine i.e., α-nucleophilic substitution. The products formed have a similar UV pattern to that of products from S^4-DNPthiothymidine but with a slightly longer wavelength (about 10nm longer).

The above results allow us to conclude that the thiol group of S^4-BrdU could be specifically modified by a suitable reagent such as FDNB and the intermediate could be used for cross-linking with various thiol nucleophiles. Using S^4-BrdU could be an advantage over 4-thiothymidine because it can be recognised by monoclonal antibodies. This might help to identify the cross-link in cellular DNA.

CHAPTER 4

DNA STUDIES

4 DNA studies

Oligonucleotides containing modified bases are used in a variety of areas such as studies of carcinogenic compounds, investigations on DNA-RNA activity and DNA-protein interactions [114]. In the context of this thesis the modified nucleosides can be considered as 'convertible' nucleosides [115] and the process of substituting them with the desired groups has been termed as 'post synthetic' substitution' [103].

One approach to site-specific chemical cross-linking of DNA was initially explored by Xu [88] in which a novel method to chemically crosslink cysteine or peptides containing cysteine with reactive DNA containing 6-methylsulphoxypurine in a site specific manner was described [88]. Some difficulties were encountered in producing 'clean' products with thiopurines. The very promising cross-linking results reported in the previous chapters with nucleosides encouraged us to explore the use of thiopyrimidines for oligomer studies. The first step was to synthesise an oligomer incorporating 4-thiothymidine.

4.1 Synthesis of oligonucleotides:

Chemical synthesis of DNA was started in 1950s in Sir Alexander Todd's lab in Cambridge. In the late 1960s Khorana and his co-workers developed phosphodiester chemistry for the synthesis of oligomers. Purification of oligomers was difficult with this method. In mid 1980s the Merrifield solid-phase synthesis method was adapted for the synthesis of DNA. With this method the purification of intermediates was eliminated. The solid phase method was then combined with fast reacting phosphoramidite approach developed by Caruthers, in which each cycle is shortened to a few minutes. Automated DNA synthesis with phosphoramidite chemistry is now the method of choice and its use has shaped modern face of biotechnology. The

chemistry of synthesising short sequences of DNA has reached an advanced stage. Numerous synthetic oligomers are produced by DNA synthesisers and used as probes for interaction with proteins, as primers for polymerase chain reaction (PCR) and for various other studies. However the synthesis of base-modified DNA remains challenging and various groups are seeking to develop better methods [116].

4.2 Synthetic Chemistry of Unmodified DNA:

The structures and chemistry of deoxynucleoside phosphoramidite monomers are shown in Figure 4.1. As discussed earlier for DNA (in Chapter-1), the monomers are also made of three parts: base, sugar and phosphoramidite. These monomers are commercially available and directly employed for the synthesis of DNA by automated DNA synthesisers [116].

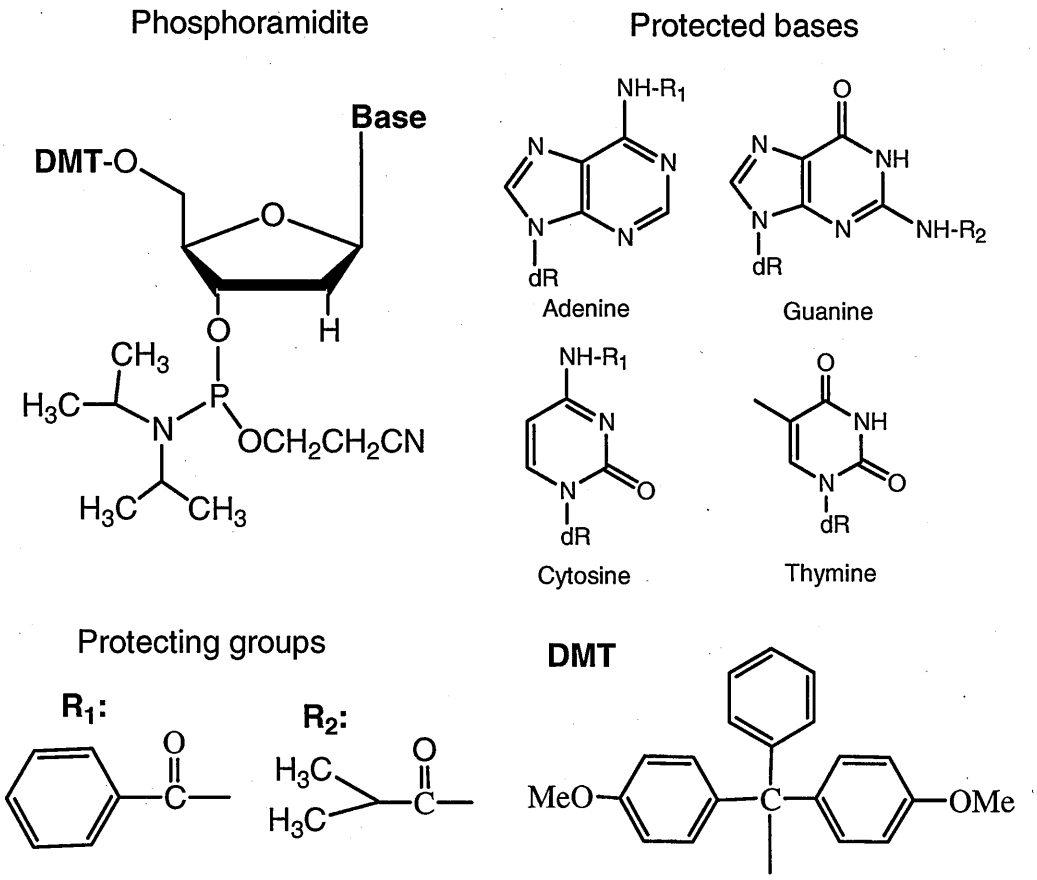


Figure 4.1: Phosphoramadite monomers of Standard Bases

(figure adapted from reference [116]).

(a) Bases

The standard bases A, G and C have amino group that requires protection. Acyl groups are used for this purpose. A benzoyl group is generally used for the protection of A and C and isobutyryl group for G, depending on their removal by the deprotecting agent which is usually ammonia. These protecting groups remain on the bases during the synthesis and are removed after the synthesis. Thymine does not need to be protected since it does not have free amino group on it.

(b) Sugar:

The 5′-hydroxyl group of deoxyribose is protected with the dimethoxytrityl group (DMT), which is acid-labile and readily removed during the synthesis of DNA to provide free hydroxyl group for elongation. When DMT is released an orange colour is observed which can be used for monitoring the coupling yields.

(c) Phosphoramidite:

This amidite, in the presence of tetrazole is very reactive towards the 5′-hydroxyl group, which shortens the coupling time significantly. The resultant phosphite triester is not very stable and readily oxidised by iodine/water solution to form a stable phosphate triester. The cyanoethyl group in the triester is easily removed by ammonia.

4.3 Automated DNA synthesis:

Automated DNA synthesis involves the following steps:

a) DMT-cleavage: The first nucleoside (usually the 3' end of the DNA sequence) is attached to a controlled porous glass or high cross-linked polystyrene support. The support can be cleaved by ammonia solution in the final deprotection step. The 5'-OH of the nucleoside is protected with the DMT group. During this step dichloroacetic

acid is used to remove the DMT group and the deprotected 5'-hydroxyl group is ready for coupling

b) Coupling: During this step the free hydroxyl group reacts with the incoming phosphoramidite monomer with activation by tetrazole. The coupling yields are over 98% and are achieved by using excess phosphoramidite monomer.

c) Oxidation: As the newly formed phosphite triester is unstable, it is converted into a stable phosphate triester by oxidation using iodine/water solution.

d) Capping: Since the coupling yield is always below 100%, the un-coupled hydroxyl groups are capped to prevent them from reacting with the phosphoramidite during the next cycle. In this capping step, acetic anhydride is often used to cap the free hydroxyl groups.

Up to this step, a cycle of elongation is completed, and then DNA synthesiser carries out successive cycles until the whole sequence of the oligomer is made as shown in figure 4.2

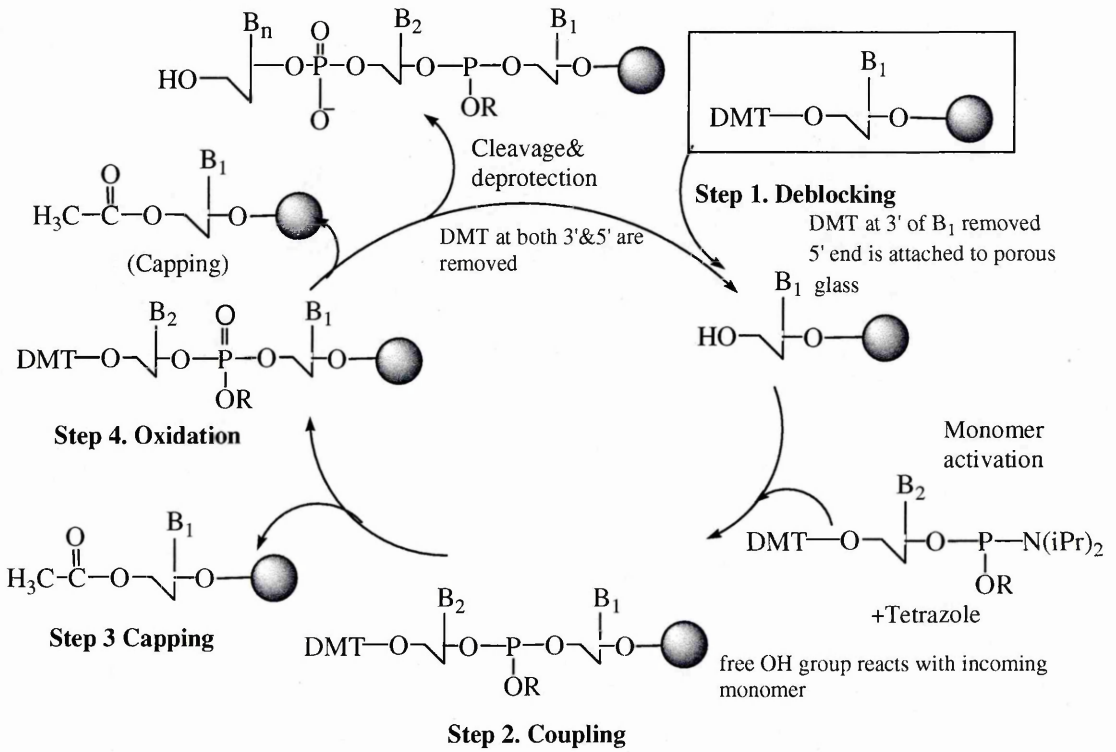


Figure 4.2 Schematic representation of automated DNA synthesis

The automated synthesis is usually carried out by using a standard protocol (details of which are usually supplied along with the automated synthesiser).

4.4 Synthesis of base-modified DNA:

(a) Preparation of base-modified nucleosides

Conventionally two major approaches were followed. One approach was to synthesise a modified base, and then to join the base with suitably protected deoxyribose moiety via chemical coupling or enzymatic method to produce the base modified nucleoside. The other approach is direct modification of naturally occurring nucleoside.

(b) Conversion of base-modified nucleoside into base modified phosphoramidite

As mentioned earlier the exocyclic amino group of the base-modified nucleoside has to be protected for A, G, C with a suitable group. Because thymidine has no exocyclic amino group it does not have to be protected. The 5′-OH of the nucleoside is selectively protected using dimethoxytrityl chloride (DMTCl) in pyridine. Basic conditions are to be maintained as the DMT group is very acid labile as mentioned earlier. The 5′-O-DMT protected nucleoside reacts with a phosphitylating agent such as 2-cyanoethyl-N,N,N′,N′-tetraisopropyl phosphoramidite with tetrazole to produce the desired monomer. The monomers are stable as solid and can be stored for a long time. The dissolved monomer is not stable after a period of two weeks and should not be used on automated synthesiser.

(c) Incorporation of the modified phosphoramidite into DNA oligomers by synthesiser

In theory the protocols used for standard monomers can be applied for the incorporation of base-modified phosphoramidites. But in practice the reactivity of the

base-modified phosphoramidites could vary due to the nature of the modified base and the protecting groups on it, therefore in most cases extra coupling times are required to achieve the optimal coupling yields for the base modified monomers. For this purpose slight changes to the standard protocol were used for unmodified monomers.

4.5 Modification of protocol

A model pentamer containing CGXTA would be synthesised where X being 4-thiothymidine. The monomer of T^s is now available commercially (Glen Research, USA). This was synthesised using the above mentioned standard protocol. The yield was low for 'X' i.e., for 4-thiothymidine (less than 60%) when compared to normal monomers. Reports [116] suggest that the reactivity of modified base monomer is generally low. The lesser reactivity could be generally due to the fact that there is not enough time to react and the reagents do not mix properly. So for cycle 'X' in the standard protocol the coupling time (step-2) was increased. The idea behind this could be that the modified monomer would have sufficient reaction time with the previous base, thus increasing the coupling yield.

Since the controlled porous glass (CPG) column used for the reactions is very narrow the reagents does not mix properly inside. So it requires a manual push and pull step with a syringe to allow thorough mixing of monomers to get better yields. So the protocol was modified in such a way that it would enable sufficient time for the reaction and manual mixing with a syringe. The steps modified are shown in bold in the table below:

MODIFIED PROTOCOL

```
/* ------------------------------------------------------------------------
/*      Function                Mode    Amount    Time(sec)      Description
/*                              /Arg1   /Arg2
/* ------------------------------------------------------------------------
$Deblocking
 144 /*Index Fract. Coll.      */ NA      1          0        "Event out ON"
  0 /*Default                 */ WAIT    0          1.5      "Wait"
 141 /*Trityl Mon. On/Off     */ NA      1          1        "START data collection"
  16 /*Dblk                   */ PULSE   10         0        "Dblk to column"
  16 /*Dblk                   */ PULSE   50         49       "Deblock"
  38 /*Diverted Wsh A         */ PULSE   40         0        "Flush sys with Wsh A"
 141 /*Trityl Mon. On/Off     */ NA      0          1        "STOP data collection"
  38 /*Diverted Wsh A         */ PULSE   40         1        "Flush system with Wsh A"
 144 /*Index Fract. Coll      */ NA      2          0        "Event out OFF"
$Coupling
   1 /*Wsh                    */ PULSE   5          0        "Flush system with Wsh"
   2 /*Act                    */ PULSE   12         0        "Flush system with Act"
   0 /*Default                */ WAIT    0          30       "Add the X monomer"
   0 /*Default                */ WAIT    0          240      "Push and Pull"
   0 /*Default                */ WAIT    0          30       "Put Column back"
   2 /*Act                    */ PULSE   4          32       "Couple monomer"
   1 /*Wsh                    */ PULSE   7          56       "Couple monomer"
   1 /*Wsh                    */ PULSE   8          0        "Flush system with Wsh"
$Capping
```

12 /*Wsh A	*/ PULSE	20	0	"Flush system with Wsh A"
13 /*Caps	*/ PULSE	8	0	"Caps to column"
12 /*Wsh A	*/ PULSE	6	15	"Cap"
12 /*Wsh A	*/ PULSE	14	0	"Flush system with Wsh A"
$Oxidizing				
15 /*Ox	*/ PULSE	15	0	"Ox to column"
12 /*Wsh A	*/ PULSE	15	0	"Flush system with Wsh A"
$Capping				
13 /*Caps	*/ PULSE	7	0	"Caps to column"
12 /*Wsh A	*/ PULSE	30	0	"End of cycle wash"

There are two advantages of doing this:

a) the modified monomer is given enough time to react with the previous nucleoside and

b) because the monomer is expensive only the required amount of monomer needs to be diluted to avoid the waste of the monomer since monomers are not stable in solution for more than two weeks as discussed earlier.

Higher tritylation yields for T^S monomer (X) were observed when compared to normal monomers (C, T and G) as shown in the figure 4.3. Higher tritylation yields for (X) modified monomers were also observed using the modified protocol in comparison to the suppliers method (tritylation yields when suppliers protocol was used not shown).

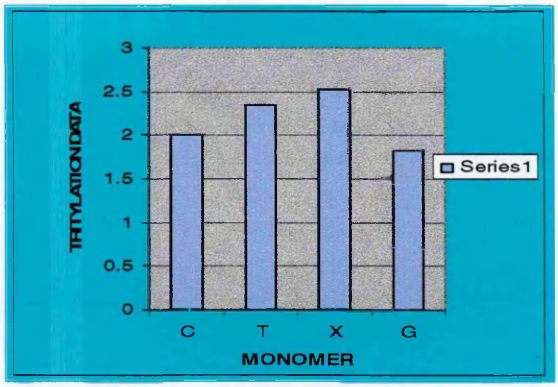


Figure 4.3 Tritylation data for pentamer using modified protocol

A model 12mer was also synthesised to see if the protocol can be used for longer modified sequences as shown in figure 4.4.

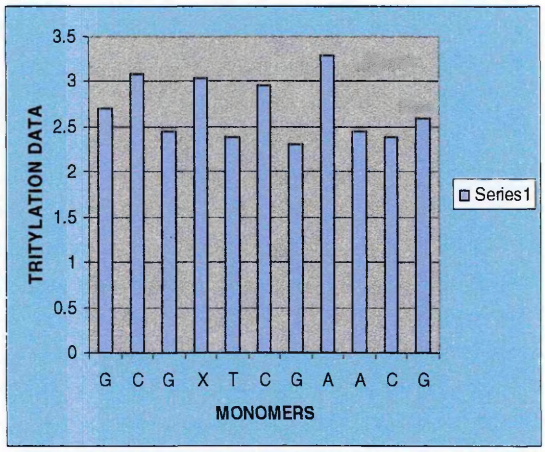


Figure 4.4 tritylation data for 12mer sequence. This sequence was synthesised using the same modified protocol for figure 4.3

High tritylation yields were obtained using the modified protocol even for the longer oligomers (in this case 12mer). So the modified protocol enhances the tritylation yield for both shorter and longer sequences.

4.6 HPLC Purification:

Two types of HPLC, reversed phase and anion exchange, are commonly used for the separation of short oligomers. When an oligonucleotide is of medium length, reverse phase HPLC is not always effective in separating the impurities from the sample. Anion exchange HPLC at neutral pH can separate oligomers differing in length by only one nucleotide with good resolution. However, to separate an oligomer of interest with modified bases from other oligomers of the same length is difficult to achieve. Xu et al have described a general procedure for the purification of synthetic oligomers of medium length in particular, containing 4-substituted thymine and 6-substituted guanine from oligomers of the same length [116]. This general protocol was followed for the purification of our synthesised oligomers.

The oligomers (pentamer and 12mer) that were synthesised previously (fig 4.3 & 4.4) are subjected to purification. The oligomer was first purified on Nensorb™ preparative cartridge. Then it was purified by preparative HPLC. A pure oligomer was obtained as shown in figure 4.5 (a).

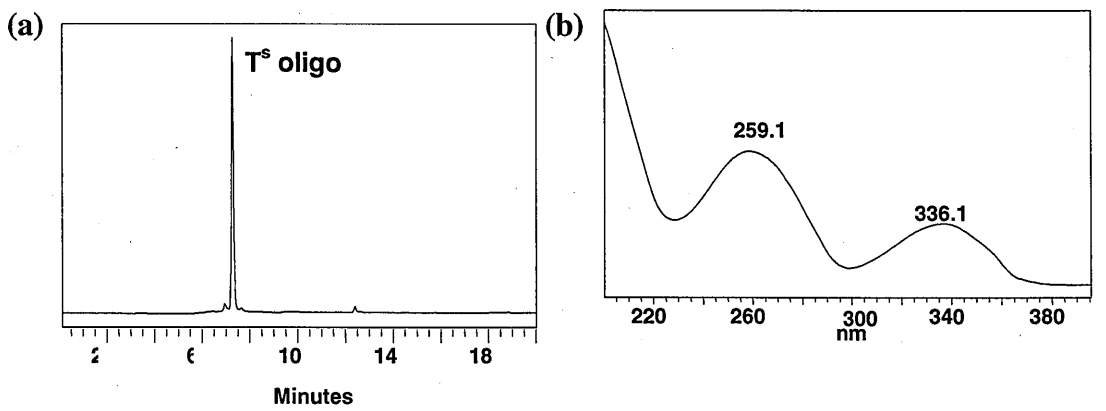


Figure 4.5 : (a) T^s incorporated oligo was synthesised using modified protocol (sequence ACTXG)

(b) UV of the oligo

The UV λ_{max} of the above peak shows presence of the modified base, T^S as it has absorption at 330-340nm. The UV was compared with the UV of T^S nucleoside (Figure 4.6).

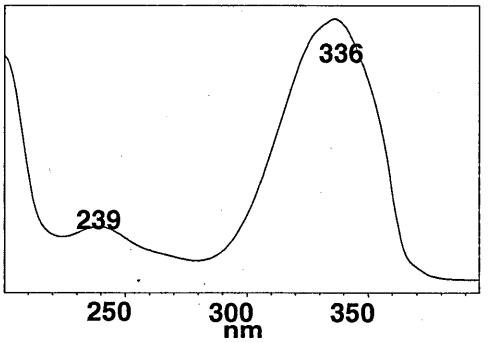


Figure 4.6: UV λ_{max} of T^S nucleoside

(Observe the difference in 1st and 2nd crusts)

4.7 Characterisation of the T^S pentamer:

The incorporation of the T^S nucleoside into the pentamer could be confirmed by nucleoside composition analysis. The above HPLC-isolated pentamer was subjected to enzymatic digestion and five peaks were observed. The new peaks were identified by injecting the standard nucleosides.

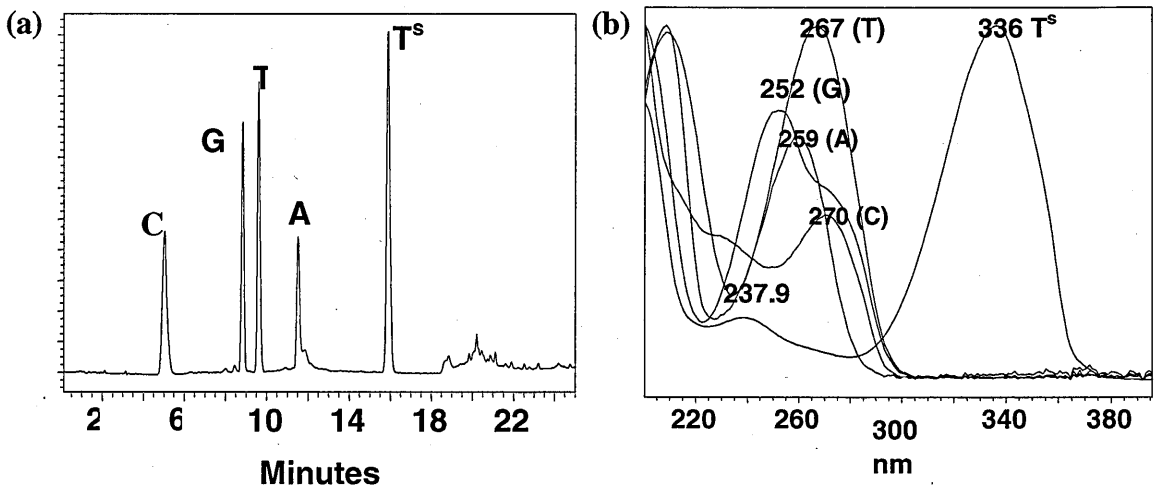


Figure 4.7: (a) Standard nucleosides were injected together (b) UV λ_{max} of standard nucleosides

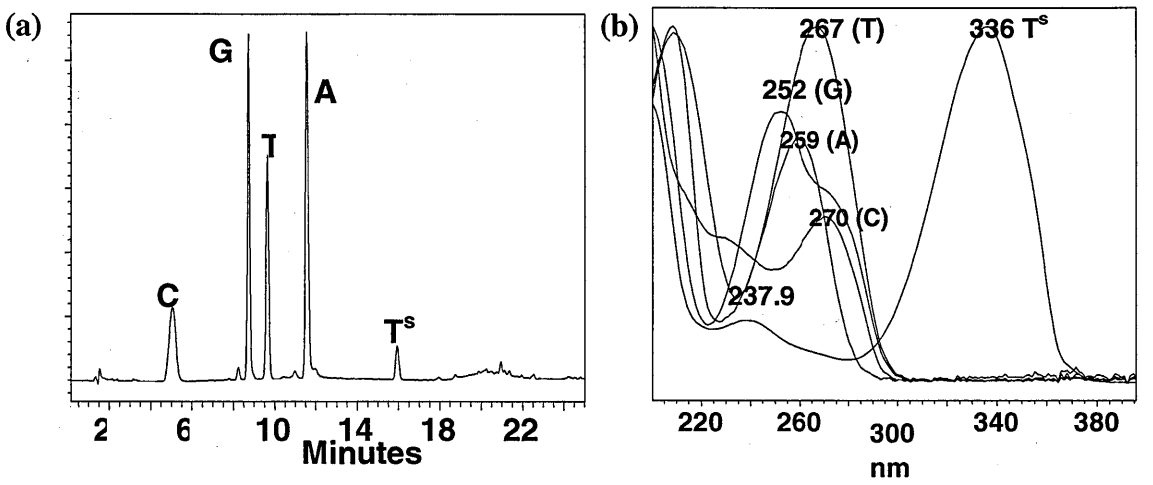


Figure 4.8: HPLC profile of nucleoside composition analysis of the pentamer containing 4-thiothymidine. The pentamer (ACTXG) was digested with Nuclease P1 and phophodiesterase for 30 minutes. The peak X has the same retention time as T^s standard.

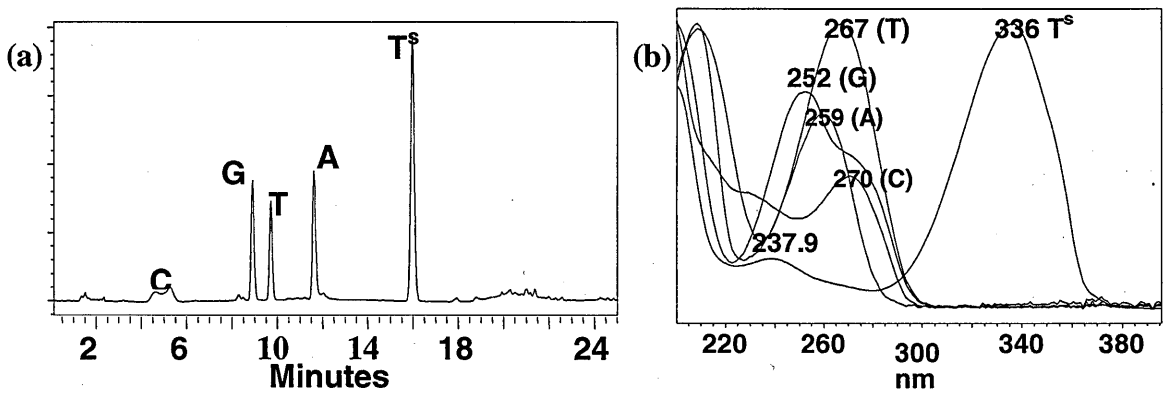


Figure 4.9: The peak X co-elute with synthesised T^s upon co-injection.

This confirms that 4-thiothymidine has been incorporated into the oligomer. This oligomer containing 4-thiothymidine can be used for site specific cross-linking.

4.8 Incorporation of S^4-BrdU into oligomer

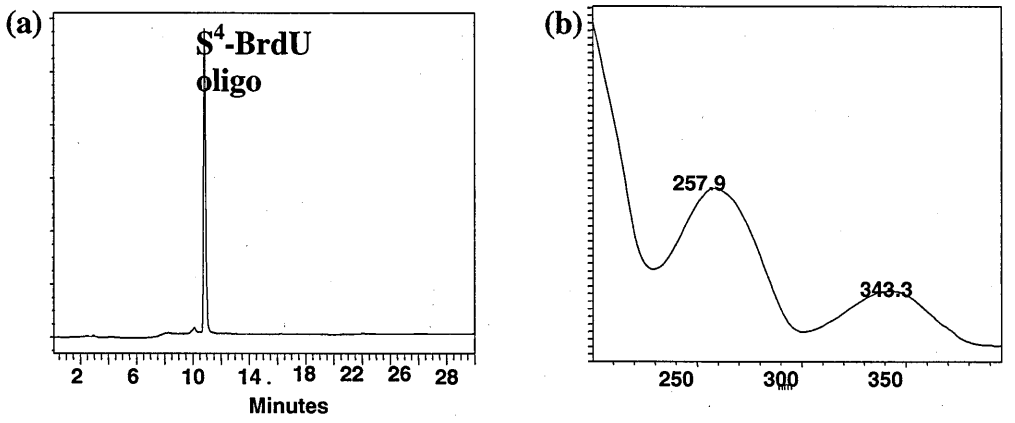


Figure 4.10: A pentamer containing S^4-BrdU was synthesised. AGXTC where, X is S^4-BrdU

S^4-BrdU was incorporated into the oligo and was isolated as a single peak as shown in figure 4.10.

4.9 Activation of modified oligomers

The thiol of the modified oligomers could be activated by a suitable reagent such as fluorodinitrobenzene. This would produce S^4-2,4 dinitrophenyl containing oligo. This group could be replaced by various incoming nucleophiles such as cysteine and glutathione. However, we had the difficulty of incorporation dinitrophyenyl group in the oligo. This could be due to the fact that Sanger's reagent is not soluble in water. We tried to overcome this problem by synthesising a water soluble reagent that can produce a stable and good leaving group when reacted with T^S containing oligo. We have chosen diflourodinitrobenzene and substituted one of the fluorine with glutathione (was chosen because it was very soluble in water). However our efforts did not produce a reagent that can still react with 4-thiothymidine and produce stable and good leaving group.

CHAPTER 5

OVERALL CONCLUSIONS AND FUTURE PERSPECTIVES

5. Overall conclusions and future perspectives

The overall objective of this project was to make use of the properties of 4-thiothymidines to introduce reactive centres into DNA that facilitate cross-linking. At the level of individual nucleosides the required chemistry was shown to be feasible and some novel reactions with likely nucleophiles were studied.

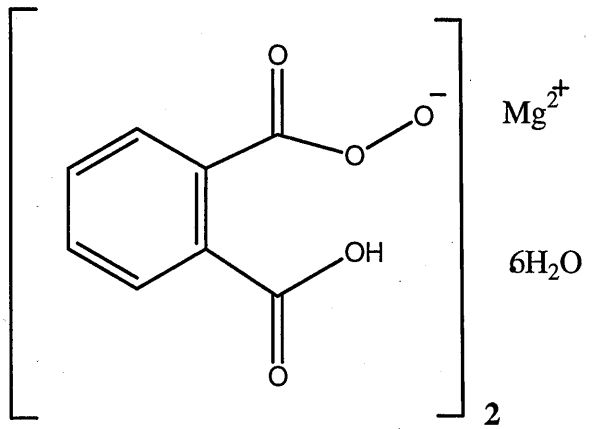
Thiol group of 4-thiothymidine and 4-thio-5-bromo-deoxyuridine can be site specifically activated with a suitable reagent such as 1-fluoro-2,4-dinitrobenzene (FDNB, Sanger reagent). The activated products S^4-2,4-dinitrophenylthiothymidine and S^4-2,4-dinitrophenylbromodeoxyuridine can be easily separated from the starting materials 4-thiothymidine and 4-thio-5-bromodeoxyuridine. The isolated products are stable in solution at neutral pH but can undergo hydrolysis at acidic and basic pH. It was shown that both S^4-2,4-dinitrophenylthiothymidine and S^4-2,4-dinitrophenylbromodeoxyuridine can be substituted by various thio nucleophiles such as mercaptoethanol, ethanethiol, cysteine and glutathione but not by the amino group of glycine under similar conditions. These results underline the potential selectivity that can be achieved as thiol groups occur much more rarely in proteins than amino groups.

The synthesis of oligodeoxynucleotides containing T^S and S^4-BrdU was successfully achieved and this demonstrated the feasibility of selectively incorporating thiothymidines into DNA. Attempts to activate the thio function in oligomers was markedly less successful that in isolated nucleosides and it appears that, this is due to the insoluble nature of the Sanger's reagent in water or the repulsion of the fluorine by phosphate.

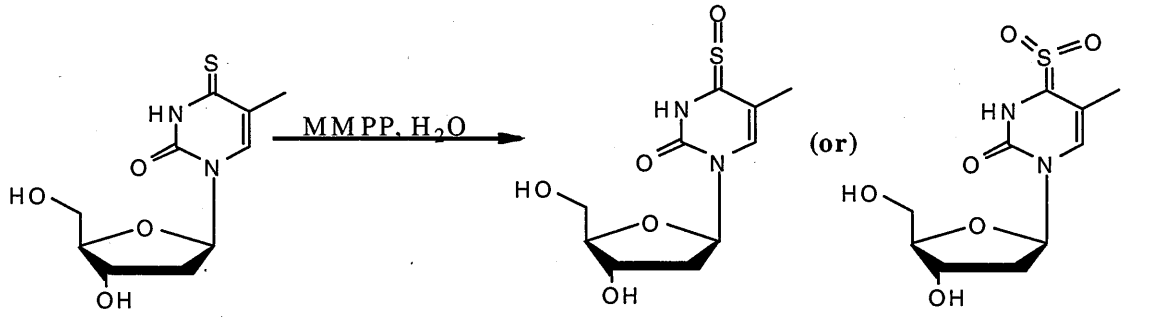
Activation of thiol of T^S containing oligo

The future prospective for this project would be to explore the possibility of cross-linking of thiols with the DNA. In order to achieve this, first the thiol group of thiopyrimidine containing oligo has to be activated with a suitable reagent like FDNB. But selection of a more biological, water soluble, mild reagent would be a better choice compared to FDNB. The possibility of selective oxidation of thiol with mild, water soluble, oxidative reagent such as magnesium monoperoxyphthalate could be explored.

Magnesium bis (monoperoxyphthalate) hexahydrate (monoperoxyphthalic acid magnesium salt or MMPP)



MMPP is a mild oxidizing reagent which is used in many types of oxidation reactions. MMPP displays low toxicity when compared to other oxidizing agents such as peroxycarboxylic acids. MMPP could oxidize 4-thiothymidine in water to form oxidized form of 4-thiothymidine. The product could be sulfoxide or sulfone form of 4-thiothymidine.



4-thiothymidine **sulphoxide form of 4-thiothymidine** **sulphone form of 4-thiothymidine**

Once the selective oxidation has been achieved it should be replaceable by simple thiol agent such as mercaptoethanol, simple but unique amino acid like cysteine and a peptide like glutathione. Once this has been achieved one should look at the most interesting aspect and that is protein linking.

Protein –nucleoside cross-link

Protein cross-linking with the nucleoside could be explored first. For this purpose a protein containing thiol group like protein disulfide isomerase (PDI) could be chosen. PDI is a 57-kDa protein that resides in the endoplasmic reticulum. PDI acts as a catalyst in the formation, reduction and isomerisation of disulfide bonds in newly synthesised proteins[117]. PDI has five distinct structural domains out of which the catalytic activity is carried out by the active-site sequence Cys-Gly-His-Cys. So the thiol group of this cysteine moiety could be used to cross-link with the activated thiol of thiopyrimidines. This would give the opportunity to explore the interaction of proteins with nucleoside or DNA (oligo).

CHAPTER 6

MATERIALS AND METHODS

6 Materials and methods

Chemicals and solvents

Chemicals and solvents were purchased most of the times from either Aldrich or Sigma and were used directly without further purification.

Instruments:

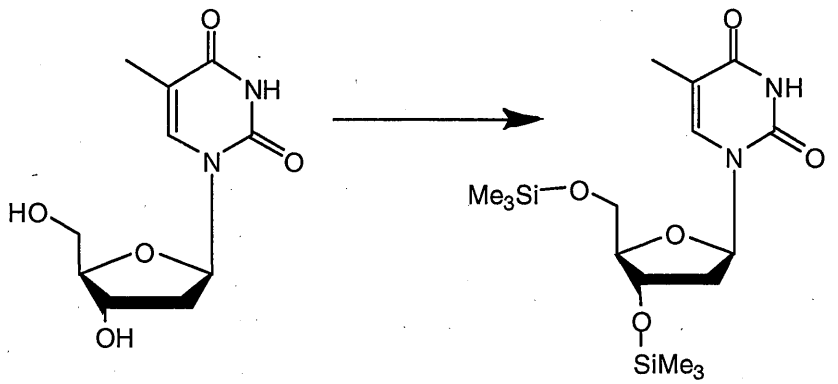
TLC: Nucleosides on TLC were identified by using p-anisaldehyde /ethanol/H_2SO_4 (5:90:5) reagent converting the nucleosides into black spots on heating.

HPLC: Waters 2690 with Photodiode array detector (Water 996)

NMR: JNM-LA 300 with FT NMR system

6.1 Synthesis of 4-thiothymidine

6.1.1 Protection of 3′ and 5′ OH groups of 1

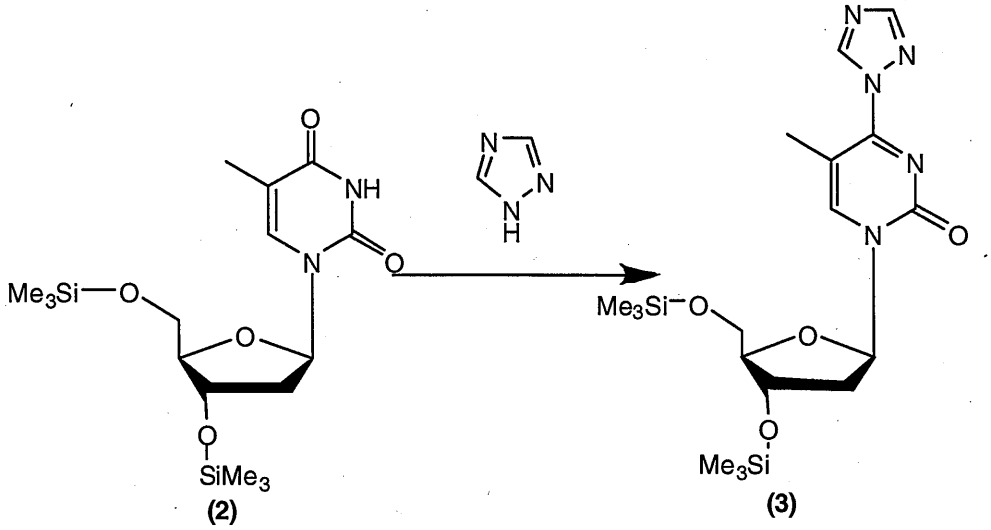


(1) (2)

2'-deoxythymidine (**1**, 5.0g, 20.6mmol) was dissolved in dry THF (150ml). Triethylamine (63.4ml, 0.454mmol) was added followed by addition of trimethylchlorosilane (6.34ml, 81.2mmol). The reaction mixture was stirred in an ice bath, with continuous stirring for an additional 2.5 hours after all the materials were

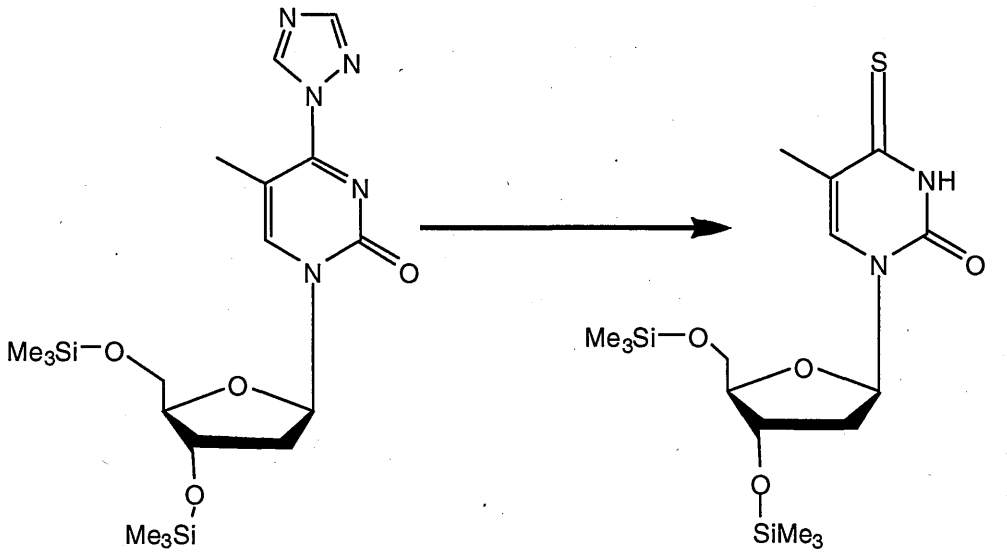
added. TLC showed no starting material. The reaction mixture was diluted with 50% ethyl acetate in hexane (250ml), washed with water (2 x 100ml) and saturated aqueous sodium chloride (80ml). The combined organic phase was dried (Na_2SO_4) and evaporated to give the protected thymidine (**2**, 3′, 5′-bis-trimethylsilyl-2'-deoxythymidine). Yield 65%.

6.1.2 Synthesis of 3



1,2,4-1*H* triazole (6.8g, 98.4mmol) was suspended in 80ml of anhydrous acetonitrile at 0°C. 2.1 ml of $POCl_3$, then 16ml of triethylamine was added slowly. After an hour **2** (2.6g, 6.7 mmol) in 30ml of acetonitrile was added over 30min. Then the solution was stirred for 16 hours at room temperature and reaction was monitored by TLC. The reaction mixture was filtered diluted with ethyl acetate (160ml) and washed with saturated aqueous sodium chloride. The organic layer was dried over anhydrous sodium sulphate and the solvent evaporated under reduced pressure to give **3.** Yield 60%.

6.1.3 Synthesis of 4



(3) **(4)**

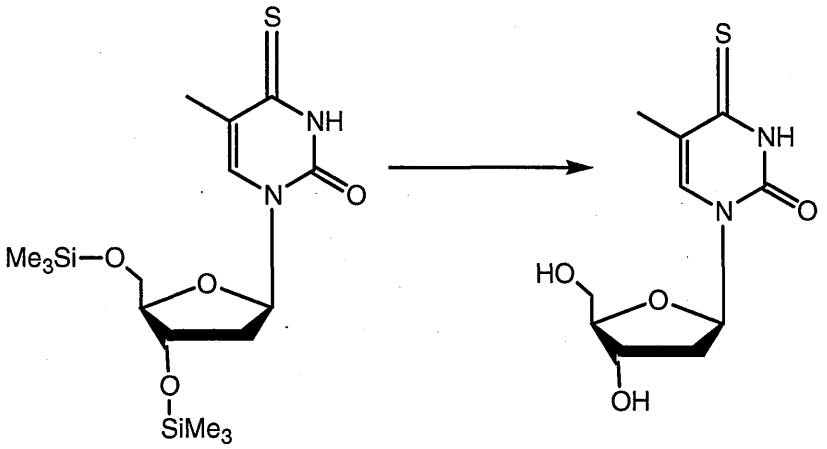
To a solution of **3** (2.62g, 6.0mmol) in 120ml of acetonitrile was added 6ml (6.39g, 83.9mmol) of thiol acetic acid in an ice water bath. The reaction was stirred overnight and monitored by TLC until the starting material converted to a new spot. The solution was diluted with dichloromethane (250ml), washed with saturated $NaHCO_3$ (2X250ml), then with saturated aqueous sodium chloride (250ml). The organic layer was dried over sodium sulphate and evaporated under reduced pressure to give crude product. Pure compound **4** was obtained by silica gel column chromatography. Yield 58%.

^{1}H NMR [DMSO-d_6] 9.18 (1H, s, 3-H of triazolo), 8.48 (1H, s, 6H)), 8.24 (1H,s, 5-H of triazolo), 6.01(1H, t, 1′H), 5.19 (1H, d, 3′-OH), 5.10 (1H, t, 5′-OH), 4.13(1H, m, 3′-H), 3.78 (1H, m, 4 ′-H), 3.59 (2H, m, 5 ′-H), 2.07-2.2 (2H, m, 2′H), 2.18 (3H, s 5-CH_3).

^{13}C NMR [DMSO-d_6] 16.2 (5-CH_3), 40.9 (C-2′), 60.4(C-5′), 69.2(C-3′), 86.9(C-1′), 88.1(C-4′), 104.4(C-5), 145.2(C-6), 147.9 (C-4), 153.1(triazole C), 153.4(triazole C), 157.7 (C-2).

6.1.4 Synthesis of 5

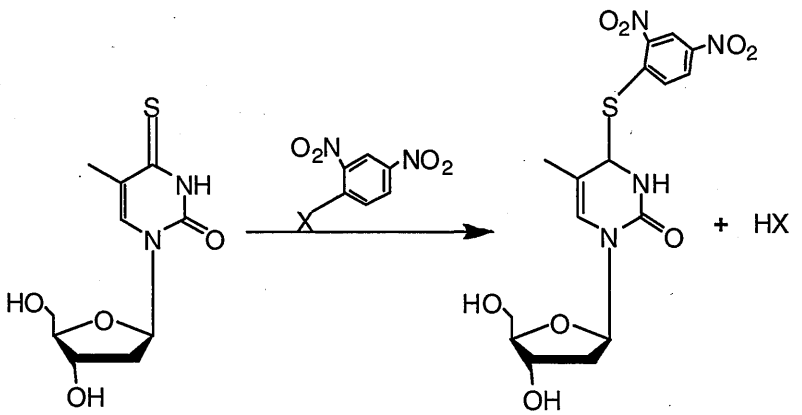
The trimethylsilyl groups are unstable in the presence of acid and 4-thiothymidine (**5**) is produced by the treatment of **4** with acetic acid in methanol.



(4) **(5)**

Addition of acetic acid deprotects 3' & 5' hydroxyl groups

6.2 Synthesis of 6.



5 **6**

(a) Reaction with ClDNB:

4-thiothymidine (14mg, 5.4μmoles) was dissolved in 1ml of acetonitrile. 12mg (5.9μmoles) of 1-Chloro 2,4-dinitro benzene was dissolved in 1ml of acetonitrile (or

DMF). 200µl of phosphate buffer (pH 9.5 1M) was added and the reagents were mixed thoroughly using a whirl mixer.

(b) Reaction with FDNB:

4-thiothymidine (14mg, 5.4µmoles) was dissolved in 1ml of acetonitrile. 8mg (4.3µmoles) of Flouro 2,4-dinitro benzene was dissolved in 1ml of acetonitrile (or DMF). 200µl of phosphate buffer (pH9.5 1M) was added and the reagents were mixed thoroughly using a whirl mixer.

(c) HPLC monitoring of the reactions

200µl of each of the above reaction mixtures were diluted with 1ml of water before injecting on HPLC. The reaction was monitored by HPLC on a PHENOMENEX, (250x4.6 mm 4µ synergy analytical column [Conditions: flow rate 0.3 mL/min. Reservoir A: H_2O; Reservoir B: CH_3CN; Reservoir C of 0.1 M phosphate buffer (pH 6.52) is kept constant at 5%; the percentage of CH_3CN was increased from 5% to 75% during the first 20 min]

When the starting material was completely disappeared, the product **6** (T^{SDNP}) at retention time 13.4 minutes was isolated by preparative HPLC.

(d) Isolation of the peak

Reversed phase preparative HPLC (Phenomenex Synergi Max 250 x 10mm column) was used for isolation **6**. The peak at retention time 13.4 minutes with λ_{max} of 314.7 nm was collected using a Waters™ 616 pump, 996 photodiode array detector and 717Plus auto-sampler. The following program was used:

Time	Flow	%B	%C	%D

0	4.00	90.0	5.0	5.0
20.00	4.00	20.0	75.0	5.0
30.00	4.00	5.0	90.0	5.0
30.01	4.00	90.0	5.0	5.0

Mobile phase B was water, mobile phase C was acetonitrile, mobile phase D was 0.1M KH_2PO_4 buffer (pH 6.6). The purified and collected **6** was re-analysed by analytic HPLC and showed a single peak.

Spectral data for T^{SDNP}

The isolated pure peak was subjected to mass spectroscopic analysis and the mass fragments were obtained under different electrospray conditions. The observed fragments were 424 M^+, 423 $(M^+-H)^+$ and 425 $(M^+H)^+$.

UV λ_{max}: 314.7 nm

6.3 Attempted synthesis of S^4–mononitrophenyl derivatives

5 (1.2mg, 4 μmoles) was dissolved in 10μl of water. 1-Fluoro-2-nitrobenzene (1.2mg, 8 μmoles) or 1-fluoro-4-nitrobenzene (1.4mg, 10 μmoles) was dissolved in 20μl of dimethyl formamide (DMF). Phosphate buffer (20μl, pH 9.5) was added to the combined solutions and the reactions were monitored by HPLC by diluting 1μl of reaction mixture to 10 with water and injecting 5μl after 1hour and 48hours

6.4 Stability of 6 in aqueous solutions:

A solution of **6** in 0.1 M phosphate buffer, pH 6.52 was mixed with an equal-volume of 1.0 M phosphate buffers of specific pH (4, 5, 6,7,8 9 and 10), then the solution was left in RT for 24 hours or 9 days at which time the solution was analysed by HPLC.

Conditions for monitoring the reactions

Waters 2690 Alliance HPLC, (996 photodiode array detector fitted) was used. Atlantis dC18 5μm 2.1X150mm column with mobile phase B as water, mobile phase C as acetonitrile, mobile phase D as 0.01M KH_2PO_4 buffer (pH 6.8) and the program used was,

Time	Flow	%B	%C	%D
0.0	0.30	90	5	5
20.0	0.30	20	75	5
20.1	0.30	90	5	5

The programme was run for 20 minutes and the time left for equilibration was 15 minutes. The sample temperature was maintained at 4°C throughout the experiment. The column temperature was maintained at 30°C.

The HPLC analysis showed three peaks with retention times 7.1 minutes (the hydrolysed product: thymidine), 10.2 minutes (the leaving group of dinitrophenylthiol) and 14.7 minutes (the starting material: S^4-2,4-dinitrophenylthiothymidine) respectively. Each sample was analysed for three times and the values were averaged.

The percentage of the degradation of **6** was calculated and the values averaged. The averaged values for the hydrolysis of T^{SDNP} and the averaged values for the thymidine formed were taken into consideration for plotting the graph.

6.5 Reaction of 6 with various nucleophiles

6.5.1 Reaction with mercaptoethanol

To a solution of of **6** (150µL [1 OD] in 0.01M phosphate buffer [pH 6.52]) was added 10µL of mercaptoethanol from a stock solution (10µL diluted with 1ml of water). Atlantis™ C_{18} 5µ (2.1x150mm) column and Waters™ 2690 with photo diode array detector were used.

Conditions: flow rate 0.3 mL/min. Reservoir A: H_2O; Reservoir B: CH_3CN; Reservoir C of 0.1 M phosphate buffer (pH 6.52) is kept constant at 5%; the percentage of CH_3CN was increased from 5% to 75% during the first 20 min, monitored at 260 nm.

The same HPLC conditions were used for co-injections with the synthesised standard and determining the characteristics of the compound 2-hydroxyethylthiothymidine.

6.5.2 Reaction with cysteine

To a solution of **6** (150µL (1 OD) in 0.01M phosphate buffer (pH 6.52)) was added 2mg (0.0164mmol) of cysteine. Atlantis™ C_{18} 5µ (2.1x150mm) column was fitted to Waters™ 2690 with photo diode array detector.

The conditions used were flow rate 0.3 mL/min. Reservoir A: H_2O; Reservoir B: CH_3CN; Reservoir C of 0.1 M phosphate buffer (pH 6.52) is kept constant at 5%; the percentage of CH_3CN was increased from 5% to 75% for 20 min, monitored at 260 nm. The sample temperature was maintained at 20° C for N^4-cysteinylthymidine (S^4-cysteinylthymidine was not observed in these conditions).

The above mentioned conditions were maintained apart from the sample temperature (which was maintained at 5°C) and all the solutions were pre-cooled before mixing. At this stage S^4-cysteinylthymidine was observed for shorter period of time.

6.5.3 Reaction of 6 with cysteine and glycine.

To a solution of 150 μL of **6** [containing 1 OD of T^{SDNP} in 0.01 M phosphate buffer (pH 6.52)] was added 16 mg (0.13 mmol) of cysteine or 56 mg (0.74 mmol) of glycine. The reactions were monitored by HPLC immediately and after 24 hours.

HPLC conditions: Atlantis™ C_{18} 5 micron (4.6 x 250mm) column; flow rate 0.3 mL/min; Reservoir A: H_2O; Reservoir B: CH_3CN; Reservoir C of 0.01 M phosphate buffer (pH 6.52) is kept constant at 5%; the percentage of CH_3CN was increased from 5% to 90% during the first 20 min, monitored at 260 nm].

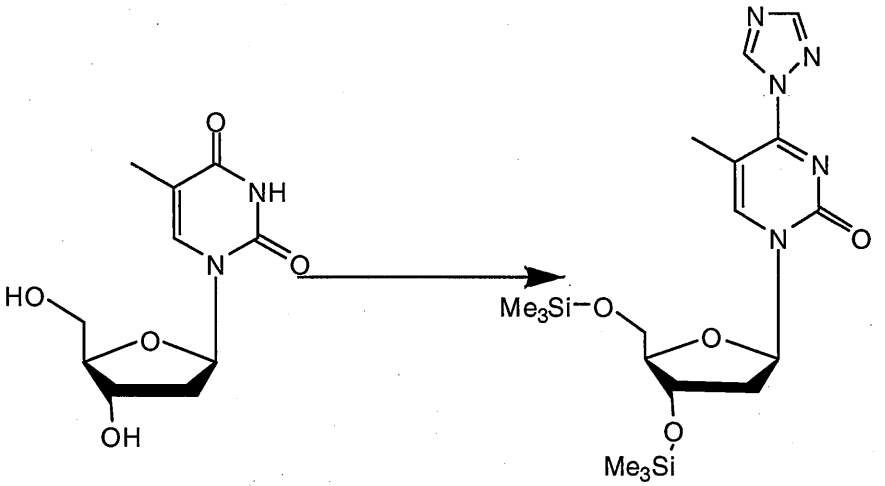
6.5.4 Reaction with glutathione

To a solution of 150μL of **6** (1 OD) of T^{SDNP} in 0.01M phosphate buffer (pH 6.52) was added 1.5mg (0.0048mmol) of glutathione. The sample temperature was maintained at 5° C. The gradient was run for 20 minutes. Phenomenex™, 2μ Synergy analytical column used. The gradient was run as follows.

	Time	flow	%A	%B	%C	%D
1	-	0.30	0.0	90.0	5.0	5.0
2	20.0	0.30	0.0	20.0	75	5.0
3	21.0	0.30	0.0	90.0	5.0	5.0
4	28.0	0.30	0.0	90.0	5.0	5.0
5	28.1	0.30	0.0	90.0	5.0	5.0

6.6 Synthesis of Standards

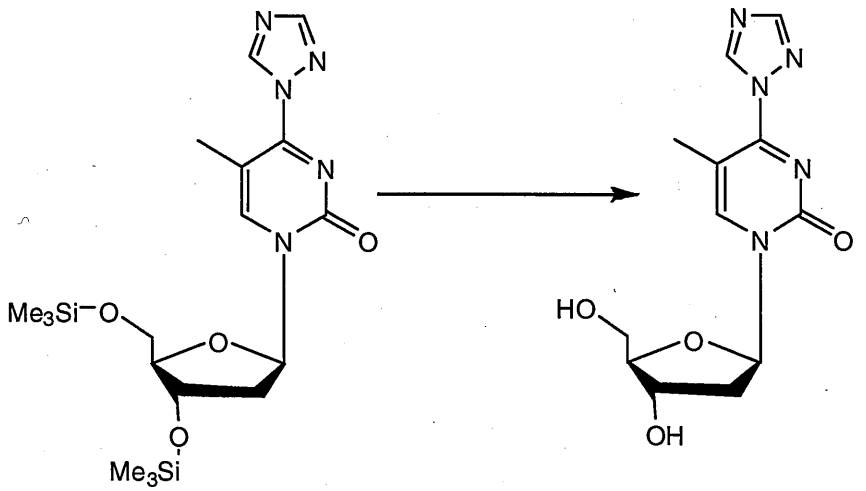
6.6.1 Synthesis of 4-triazolothymidine (11)..



(1) (3)

This synthesis was based on a previously reported protocol[99] . Thymidine (1.22g, 5.04 mmol) was suspended in dry THF (50 mL) in an ice bath. Triethylamine (11 mL, 78.26 mmol) and chlorotrimethylsilane (3.5 mL, 27.38 mmol) were added to the suspension drop by drop. The colour of the solution remains white. After 5 hours TLC showed one major spot (95%) and two minor spots (in 10% $CH_3OH/CHCl_3$). Then, the reaction mixture was cooled again (ice water bath), under continued stirring 1,2,4-1H-triazole (4g, 57.92 mmol) was added and phosphorous oxychloride (1 mL, 10.69 mmol) added dropwise. The reaction was left stirring overnight. The solution turned

to yellow, and was poured into saturated aqueous sodium hydrogen carbonate (300 mL) and resultant mixture was extracted with dichloromethane (2 x 50 mL).



(3) **(11)**

The combined organic extracts were dried ($MgSO_4$) and evaporated under reduced pressure. Acetic acid-methanol (1:4 v/v, 15 mL) was added to the residue, and the resulting solution was allowed to stand at RT overnight. Diethyl ether (30 mL) was added drop by drop, with stirring; to this solution over a period of 30 min. Colourless crystals of **11** (660 mg, yield 45%) were collected by filtration.

M.P. 175°C.

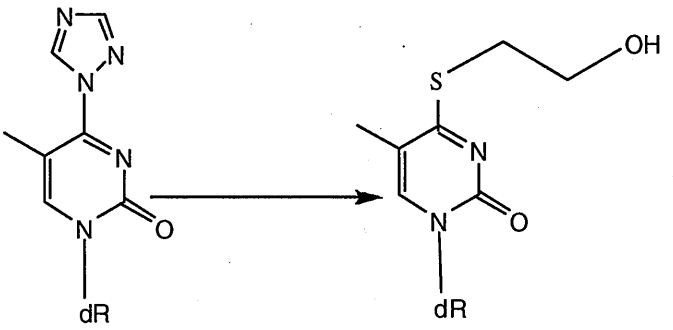
^{1}H NMR [DMSO-d_6] 9.18 (1H, s, 3-H of triazolo), 8.48 (1H, s, 6H)), 8.24 (1H,s, 5-H of triazolo), 6.01(1H, t, 1′H), 5.19 (1H, d, 3′-OH), 5.10 (1H, t, 5′-OH), 4.13(1H, m, 3′-H), 3.78 (1H, m, 4 ′-H), 3.59 (2H, m, 5 ′-H), 2.07-2.2 (2H, m, 2′H), 2.18 (3H, s 5-CH_3).

^{13}C NMR [DMSO-d_6] 16.2 (5-CH_3), 40.9 (C-2′), 60.4(C-5′), 69.2(C-3′), 86.9(C-1′), 88.1(C-4′), 104.4(C-5), 145.2(C-6), 147.9 (C-4), 153.1(triazole C), 153.4(triazole C), 157.7 (C-2).

The compound was subjected to mass spectroscopic analysis and the mass fragments were obtained under electrospray conditions. The observed fragments were:

M^+ 293, M^+-H 292 and M^+H 294.

6.6.2 Synthesis of 2-hydroxyethylthiothymidine (8):



(11) **(8)**

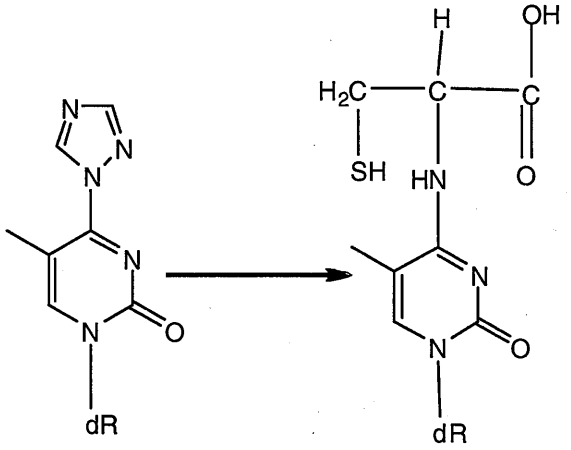
11 (100mg, 0.35mmoles) was dissolved in methanol (15ml), to which mercaptoethanol (50µl, 0.70mmoles) and triethylamine (50µl, 0.35mmoles) were added. The reaction was monitored by thin layer chromatography (TLC) using 50% methanol in chloroform. The starting material (Rf=0.34) was immediately and completely converted to a new spot with low Rf =0.20. The solvent was evaporated under reduced pressure. The residue was re-dissolved in methanol and put in a container with diethyl ether. White crystals of **8** were obtained in a yield of 80%. The product was confirmed to be β-hydroxyethylthiothymidine by spectral data:

^{1}H NMR Data (in DMSO-d_6): 1.95 (3H, s, 5-methyl), 2.01-2.28 (2H, m, 2' and 2"-H), 3.21 (2H, t, α-methylene), 3.60 (4H, m, 5'-H, and β-methylene), 3.83 (1H, dd, 4'-H), 4.21 (1H, m, 3'-H), 5.01 (1H, t, 5'-OH), 5.24 (1H, d, 3'-OH), 6.06 (1H, t, 1'-H), 7.9817 (1H, s, 6-H).

^{13}C NMR (in DMSO-d_6) 13.67 (5-CH_3), 59.35 (C-5'), 60.81 (β-C of OH), 69.77 (C-3'), 85.17 (C-1'), 87.73 (C-4'), 110.44 (C-5), 138.60 (C-2), 152.43 (C-6), 176.2 (C-2)

UV λ_{max}: 308.8; MS *m/z*: 302.88 (M) 186.9 [(M+ H) - sugar) and the mass fragments were obtained under electro spray conditions. The observed fragments were M^+ 302, M^+-H 301 and M^+H 303.

6.6.3 Synthesis of 4-cysteinylthymidine (20).



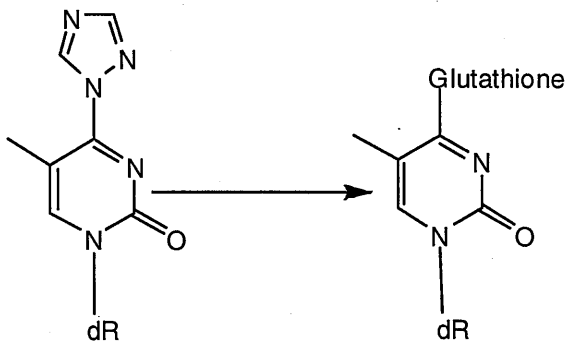
(11) **(20)**

11 (100 mg, 0.35 mmol) in 15 ml of water was treated with cysteine (100mg, 0.82mmoles) and triethylamine (50 µl, 0.35mmoles). The reaction was monitored by TLC (40% methanol in chloroform). The starting material (Rf=0.58) was completely converted to a new spot with low Rf=0.20 immediately. The solvent (water) was removed by freeze-drying. The residue was purified with a silica gel column chromatography (40% methanol in chloroform). The product was collected and re-crystallised from methanol and diethyl ether. 90mg of **20** was obtained (yield 75%).

^{1}H NMR Data (in DMSO-d_6): 1.90 (3H, s, 5-methyl), 1.95-2.10 (2H, m, 2'-H & 2''-H), 3.15 (2H, m, β-CH_2), 3.5341 (2H, m, 5'-H), 3.7410 (1H, m, 4'-H), 4.2536 (1H, m, 3'-H) 4.47 (1H, m, α-CH), 5.02 (1H, br, 5'-OH), 5.19 (1H, br, 3'-H), 6.1546 (1H, t, 1'-H), 6.8252 (1H, s, N-H), 7.6350 (1H, s, 6'-H).

^{13}C NMR Data (in DMSO-d_6): 12.77 (5-CH_3) 25.82 (β-C of linked cysteine), 45.41 (C-2'), 56.07 (α-C of linked cysteine), 61.72 (C-5'), 65.00 (No proton-attached), 70.4 (C-3'),84.63 (C-1'), 87.09 (C-4'), 101.49 (C-5), 137.80 (C-6); 154.95 (C-2), 161.80 (C-4). UV λ_{max}: 278 nm; HRMS (electrospray): *m/z* 346.1071 ([M^++1])

6.6.4 Synthesis of 4-glutathionyl-β-thiothymidine



(11) **(24)**

9 (100 mg, 0.35 mmol) was dissolved in 25 ml of water, to which glutathione (150 mg, 0.52 mmol) and triethylamine (50 µl, 0.36 mmol) were added at room temperature. The reaction was monitored by TLC (50% methanol in chloroform). The starting material was converted to a new spot immediately. The solvent (water) was removed by freeze drying. The white precipitator was obtained in quantitative yield. HPLC analysis shows a major peak (purity over 95%). Structural characterisation:

UV λ_{max}: 309nm; HRMS (electrospray): *m/z* 532.1705 ([M^++1]).

6.7 Data for DTT experiments:

DTT (6mg, 39μmoles of stock prepared and 10 μl, from the stock 0.39 μmoles) was added to peak 4. Peak 3(T^{cys}) was purified on HPLC which forms peak 4 on standing for overnight. This newly formed peak has the same UV absorption at 278 like T^{cys}.

HPLC setup:

Waters 2690 Alliance HPLC, (996 photodiode array detector fitted) was used to monitor the reaction of T^{SDNP} with Cysteine. Atlantis dC_{18} 5μm 2.1X150mm column with mobile phase A as methanol, mobile phase B as water, mobile phase C as acetonitrile, mobile phase D as 0.01M KH_2PO_4 buffer (pH 6.8) and the program used was,

Time	Flow	%B	%C	%D
0.00	0.30	95	0	5
25.00	0.30	60	35	5
26.00	0.30	25	70	5
30.00	0.30	25	70	5
31.01	0.30	95	0	5

The program was run for 25 minutes and the time left for re-equilibration was for 25minutes. The sample temperature was kept at 4°C throughout the experiment. The column temperature was maintained at 30°C.

6.8 ^{1}H and ^{13}C NMR data of modified thymidine nucleosides:

^{1}H NMR of thymidine analogues

	1'-H	2'-H	CH_3	3'-H	4'-H	5'-H	3'-OH	5'-OH	6-H	**NH**	**β-methylene**
a	6.06	2.01	1.95	4.21	3.83	3.60	5.24	5.01	7.98	**n/a**	**3.60**
b	6.15	1.95	1.90	4.25	3.74	3.53	5.19	5.02	7.63	**6.82**	
c	6.01	2.07	2.18	4.13	3.78	3.59	5.19	5.10	8.24	**n/a**	
d	6.06	2.09	N/A	4.22	3.76	3.53	5.24	5.16	8.38	**11.77**	

^{13}C NMR of thymidine analogues

	CH_3	1'-C	2'-C	3'-C	4'-C	5'-C	2-C	**4-C**	**5-C**	6-C
a	13.67	85.17	40.6	69.77	87.73	59.35	138.60	**176.2**	**110.44**	152.43
b	12.77	84.63	45.41	70.4	87.09	61.72	154.95	**161.80**	**101.49**	137.80
c	16.2	86.9	40.9	69.2	88.1	60.4	153.1	**157.7**	**104.4**	145.2
d	N/A	84.84	40.05	69.96	87.56	60.81	149.77	**159.20**	**95.69**	140.31

a)β-hydroxyethylthiothymidine:
^{1}H NMR Data (in DMSO-d_6): 1.95 (3H, s, 5-methyl), 2.01-2.28 (2H, m, 2' and 2"-H), 3.21 (2H, t, α-methylene), 3.60 (4H, m, 5'-H, and β-methylene), 3.83 (1H, dd, 4′-H), 4.21 (1H, m, 3′-H), 5.01 (1H, t, 5′-OH), 5.24 (1H, d, 3′-OH), 6.06 (1H, t, 1′-H), 7.9817 (1H, s, 6-H).
^{13}C NMR (in DMSO-d_6) 13.67 (5-CH_3), 85.17 (C-1'), 69.77 (C-3'), 87.73 (C-4'), 59.35 (C-5'), 60.81 (β-C of OH), 138.60 (C-2), 176.2 (C-4), 110.44 (C-5), 152.43 (C-6)

b)4-Cysteinyl thymidine:
^{1}H NMR (in DMSO-d_6): 1.90 (3H, s, 5-methyl), 1.95-2.10 (2H, m, 2'-H & 2″-H), 3.15 (2H, m, β-CH_2), 3.5341 (2H, m, 5'-H), 3.7410 (1H, m, 4'-H), 4.2536 (1H, m, 3'-H), 4.47 (1H, m, α-CH), 5.02 (1H, br, 5'-OH), 5.19 (1H, br, 3'-OH), 6.1546 (1H, t, 1'-H), 6.8252 (1H, s, N-H), 7.6350 (1H, s, 6'-H).
^{13}C NMR (in DMSO-d_6): 12.77 (5-CH_3) 25.82 (β-C of linked cysteine), 45.41 (C-2'), 56.07 (α-C of linked cysteine), 61.72 (C-5'), 65.00 (No proton-attached??? CO2?) 70.4 (C-3'), 84.63 (C-1'), 87.09 (C-4'), 101.49 (C-5), 137.80 (C-6); 154.95 (C-2), 161.80 (C-4)

c)1,2,4 triazolothymidine:
^{1}H NMR [DMSO-d_6] 9.18 (1H, s, 3-H of triazolo), 8.48 (1H,s, 5-H of triazolo), 8.24 (1H,s, 6H), 6.01(1H, t, 1'H), 5.10 (1H, t, 5'-OH), 5.19 (1H, d, 3'-OH), 4.13(1H, m, 3'-H), 3.78(1H, m, 4'-H), 3.59(2H, m, 5'-H), 2.07 (2H, m, 2'H), 2.18 (3H, s 5-CH_3).
^{13}C NMR [DMSO-d_6] 16.2 (CH_3), 40.9(C2'), 60.4(C5'), 69.2(C3'), 86.9(C1'), 88.1(C4'), 104.4(C5), 145.2(C6?), 147.9(triazole C), 153.1(C2?), 153.4(triazole C), 157.7 (C4?).

d)5-Bromo-2'-deoxyuridine:
^{1}H NMR [DMSO-d_6]: 2.09-2.14 (2H, m, 2'-H & 2"-H), 3.53-3.64 (2H, m, 5'H), 3.76-3.79 (1H, m, 4'H), 4.22-4.23 (1H, m, 3'H), 5.16 (1H, br, 5'OH), 5.24 (1H, br, 3'OH), 6.06-6.11 (1H, t, 1'H), 8.38 (1H, s, 6H), 11.77 (1H, br, N-H)
^{13}C NMR [DMSO-d_6] 40.05(C2'), 60.81(C5'), 69.96 (C3'), 84.84 (C1'), 87.56 (C4'), 95.69 (C5), 140.31 (C6), 149.77 (C2), 159.20 (C4).

6.9 HPLC conditions for S^4-BrdU reactions:

Column details: XTerra® MS C_{18} 3.5µm, 2.1X150mm column.

Time	Flow	%A	%B	%D
	0.20	93.0	2.0	5.0
10.00	0.20	75.0	20.0	5.0
20.00	0.20	65.0	30.0	5.0
20.01	0.20	65.0	30.0	5.0
30.00	0.20	15.0	80.0	5.0
35.00	0.20	15.0	80.0	5.0
35.01	0.20	93.0	2.0	5.0

%A Water

%B Acetonitrile

%C buffer (0.01M KH_2PO_4 buffer pH6.55)

6.10 Chemicals and enzymes for DNA synthesis and oligomer analysis:

The CPG-linked monomers and the chemicals used on the synthesiser were obtained from Cruachem (Glasgow, Scotland) and the phosphoramidite protected monomers (including 4-thiothymidine) from Glen Research Corporation. Activator solution, Cap-A, B solutions, Oxidising solution, and acetonitrile solution was obtained from Cruachem (Glasgow, Scotland). The phosphatase, alkaline from bovine intestinal mucosa and phosphodiesterase II from bovine spleen were obtained from Sigma. Acetonitrile (HPLC grade) was dried with molecular sieves for at least 48 hours. All the chemicals used for chromatographic separation and Nensorb prep were HPLC grade including water.

(a) Monomers:

The monomers are generally protected as follows:

- The 5'-hydroxyl positions are protected with DMT groups
- Phosphorous is protected with diisopropyl amine groups and a β-cyanoethyl group.
- The exocyclic amines of adenosine, guanosine and cytidine are protected with a new, stable base protecting group such as tertiary butylphenoxyacetyl (tBPA).
- Thymidine does not need to be protected.
- Monomer 4-thiothymidine (T^S) was obtained from Glen Research for the synthesis of modified oligomers. The sulphur at position 4 of thymidine is protected with cyanoethyl group and removable under basic condition.

(b) Synthesis of oligomers

5'ATTGC3' sequence was synthesised using DNA synthesiser (Expedite™ 8908). The final tritylation yields were 80%. 1ml of ammonia solution was added and left overnight to cleave the oligonucleotide from the support. DMT of the last nucleotide (in this case adenine) was left on for Nensorb™ prep cartridge purification. The oligonucleotide was purified as per the manufacturer's instruction and nine fractions of 1ml were collected into 1.5ml micro centrifuge tubes. All the samples were checked for UV absorbance. Maximum yields were obtained in fraction four and five. These fractions were diluted to 20times (50µl made up to 1ml with water) and the absorbance was taken. Fractions 4 and 5 were vacuum dried and labelled as TSR-N1 (4 & 5 respectively) for future use.

(c) Synthesis of oligomers with modified base (T^S)

5'ATXGC3' sequence was synthesised (the tritylation yields were about 60%) and purified as exactly mentioned above except for the deprotection step which was carried out with 50mM NaSH in concentrated NH_4OH for 24hrs at room temperature. Fractions 4, 5& 6 of the collections gave the maximum yield. This was diluted for 20 times (50µl of sample + 950µl of water) and UV absorbance was taken. The samples were dried under vacuum and labelled as TSR-MOD 1 (4, 5& 6 respectively) for further studies. Similarly TSR-12mer (5'CGC AAG CTX GCG 3') was synthesised and stored. 'X' in this sequence denotes 4-thiothymidine. Absorbance at 330nm was observed during UV measurement for the modified oligomers (4-thiothymidine has maximum absorption at 330nm).

6.11 Purification of oligomers

Oligodeoxynucleotides (TSR N1) 5'ATTGC3', (TSR MOD1) 5'ATXGC3' and (TSR12mer) 5'CGC AAG CTX GCG3' were synthesised by Expedite™ 8908 DNA synthesiser. The purification of oligodeoxynucleotides was carried out in two stages: first, crude synthetic oligomers with DMT on the 5'-OH of the terminal nucleotide were isolated and partially purified with Nensorb™ prep cartridges (Du Pont, U.S.A). During this stage, the failure sequences without DMT on the 5'-terminus was removed, and the sequences ATTGC, ATXGC and CGC AAG CTX GCG were then detritylated and eluted from the cartridge. These are only partially purified oligomers. HPLC was employed to obtain the oligomers of highest purity.

(a) Purification of oligomer using Nensorb™ cartridge:

Nensorb™ prep cartridges can be used for the simultaneous purification and detritylation of milligram quantities of oligonucleotides obtained from automated synthesisers. Trityl-on oligonucleotides are applied to the Nensorb prep™ cartridge in 0.1M triethylammonium acetate (TEAA), pH 7.0 or concentrated ammonium hydroxide. Salts, failure sequences, and synthetic by-products are washed away while the trityl on oligonucleotide remains bound to the cartridge. The trityl group is then hydrolysed from the 5'-end of the oligonucleotide with a 0.5% trifluoroacetic acid wash, leaving the trityl wash oligonucleotide bound to the cartridge. Subsequent recovery of the purified, trityl-off oligonucleotide is accomplished with a 35% methanol wash.

(b) Procedure for oligonucleotide purification and detritylation:

For maximum yield from Nensorb prep:

- The synthetic oligonucleotide must contain a 5' trityl group
- Drying of resin bed should be avoided during wash steps
- Maximum amount of oligonucleotide which can be purified and detritylated is 50 O.D units (1mg)

(c) Sample Preparation

1. After automated synthesis and deblocking with ammonium hydroxide the oligonucleotide must be left in the DMT (trityl-on) state.
2. The oligonucleotide is loaded directly in concentrated NH_4OH if lower recoveries can be tolerated.

(d) Cartridge Preparation

1. Clamp the Nensorb Prep cartridge to a secure support.

2. Attach one tubing adaptor to the Nensorb prep cartridge and the other to a plastic syringe.

3. Activate with 10ml of methanol. Flow rate not to exceed 10ml/min while pulling solvent through the column.

4. Pre equilibrate the cartridge with 5ml 0.1M TEAA, pH 7.0

(e) Oligonucleotide purification

1. Pipette the sample, which has been dissolved in 4ml of 0.1M TEAA, pH 7.0, on the top of resin bed. Pull the sample through resin bed until meniscus reaches top of the bed.
2. Wash the cartridge with 10ml of acetonitrile/0.1 M TEAA pH 7.0 (1:9). Failure sequences, salts, and synthetic by-products are washed away in this step while the trityl on oligonucleotide remains bound to the cartridge.

(f) Detritylation and recovery of the purified trityl off oligonucleotide

1. Wash cartridge with 25ml of 0.5% TFA. The trityl group is hydrolysed in this step with the trityl-off oligonucleotide remaining bound to the resin.
2. Wash cartridge with 10ml of 0.1M TEAA, pH 7.0 to remove acid.
3. Replace 20ml syringe with a 3ml plastic syringe. Add 10ml of 35% methanol/water to the cartridge. Pull 1ml fraction to a 1.5ml micro centrifuge tube. Collect 10 fractions.

4. Check the O.D of each fraction to locate the purified trityl off oligonucleotide. It is usually found in fractions 3 to 6 with majority at 4 or 5
5. Dry the desired fractions by lyophilization or vacuum centrifugation. The oligonucleotide should be stored dry and is reconstituted immediately before use.

6.12 HPLC Conditions for purification

Both analytical (Phenomenex Luna 5μ, C18 (2), 25cm/4.6mm ID) and semi preparative (Phenomenex synergi 25cm/10mm ID) columns were used to purify modified and normal pentamers. Analytical column was fitted to Varian 9065 instrument and semi preparative to Varian 5000. For analytical purpose gradients were formed from 0.01M aqueous KH_2PO_4 (pH 6.5) (buffer A with 2% CH_3CN) and 0.01M aqueous KH_2PO_4 (pH6.5) (buffer B with 40% CH_3CN) at a flow rate of 1ml/min. For preparative procedure gradients were formed from 0.1M TEAA (pH 6.8) (buffer A with 2% CH_3CN) and 0.1M TEAA (pH 6.8) (buffer B with 40% CH_3CN). Oligomers were further purified and vacuum dried.

6.13 Nucleoside composition analysis

The purity of the oligomers can be assessed by analysis of nucleoside digest. Generally, 0.5 A_{260} unit of oligomer was dissolved in 160μL of H_2O and 20μL of 600mM Tris-HCl, 60mM $MgCl_2$, and pH 8.5. Nuclease P1 (10μL, 10μg of protein) was added and the mixture incubated (37°C, 30 min), and then alkaline phophatase (10μL, 5μg of protein) was added and incubation continued for 30min. The deoxyribonucleosides were separated by HPLC. The chromatography was monitored at 260nm for the detection of dC, dG, T and dA and at 335nm for 4-thiothymidine.

There was very good absorbance at 335nm, which confirms the presence of 4-thio thymidine in the oligomer ATXGC.

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APPENDIX

BIOL 374-Thiothymidine as a potent pro-drug and its use for DNA crosslinking

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listinctive photochemical properties of DNA thiobases have not been extensively evaluated for their therapeutic potential (Favre et al; 'hotochem Photobiol B 1998,42:109-124). Recently our group (Massey et al; Current Biology, 2001, 11:1142-1146) reported the ɔration of thiopyrimidine nucleoside, 4-thiothymidine as a pro-drug into DNA of cancerous cells and the use of nonlethal UVA doses to ıem. This thiobase/UVA treatment may offer a new therapeutic approach for non-malignant conditions like psoriasis or for superficial mors that are accessible to phototherapy. This type of therapy has an advantage over the conventional therapies as it targets the ous cells selectively. More recently, we also reported the chemical synthesis and incorporation of (4-thiothymidine analogue) pro-drug 5-bromo-2′-deoxyuridine (Xu et al; Bioorganic and medicinal chemistry letters, 2004, 14:995-997). While investigating the mechanism toxicity of these pro-drugs we have found that the thio group of 4-thiothymidine can be selectively activated to a stable but readily aced functional group. This can be readily converted to desired targets, thus offering a novel synthetic route to 4-modified thymidine ıgues and a valuable tool for chemical manipulation including site specific crosslinkings with other macromolecules, DNA or proteins.

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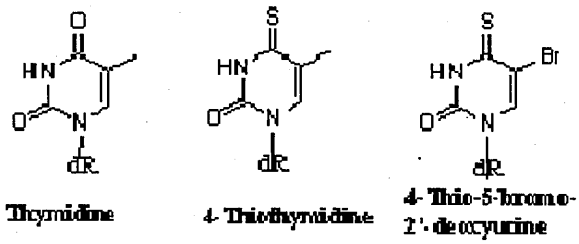

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